

Which accelerator will be next?

The possibility that the Superconducting Super Collider will be cancelled, or at least seriously delayed, and impending developments in Europe, suggest the time has come for the United States to join CERN at Geneva.

As with much that happens in the US Congress, last month's defeat in the House of Representatives of the proposal to continue supporting the Superconducting Super Collider (SSC) does not imply finality. The Senate this week may begin to lay the groundwork for a compromise with the House from which SSC may yet rise like a phoenix. But this is only the first of half-a-dozen annual battles the accelerator's supporters will have to fight before the machine is built. The chance that the SSC will survive all of them cannot be high. Although the passage of time (and the spending of dollars) will make outright cancellation seem that much more galling, alarm about the federal deficit could make even that palatable. A contribution to the cost from Japan of \$1.5 billion or thereabouts could make a decisive difference in Washington, but the Japanese high-energy physics community has set its heart on different things (see page 266).

That is why the world's high-energy physics community, fond of boasting (justly) that it is the best-integrated of all, should seize this chance to reconsider its future. In Europe, high-energy physicists have long acknowledged that new accelerators are far too expensive (and short-lived in competitive usefulness) to be supported nationally. (That is not to diminish the importance of Germany's DESY at Hamburg — a national facility put to international use.) The same principle seems to be accepted in the United States, but as an unwelcome stratagem for the future, for "the accelerator after next". What will happen if the next accelerator, SSC, is never finished?

What this means is that the high-energy physics community internationally should recognize that the time for an international accelerator-building venture is not tomorrow but today, in the here and now. As good luck will have it, the present is full of opportunities that will not recur this century. Later this year, the council of the European high-energy physics laboratory (CERN) at Geneva will have to decide in principle to build the its Large Hadron (proton-antiproton) Collider (LHC) in the tunnel of the electron-positron collider called LEP. The same council will have to choose a new director for the laboratory, in succession to Dr Carlo Rubbia, who will be leaving at the end of 1993.

The CERN machine will not be as powerful, in maximum energy and luminosity, as SSC is planned to be, but it should cover much of the same ground and should be operating sooner. In an orderly world, LHC would have been run for a little while before the final decisions were made about SSC. In the event, when it has run for a little while, it may become

clear that a different kind of accelerator, perhaps two oppositely directed linear accelerators of electrons, would be the best next step, in which case both the US Congress and the taxpayers whose funds it spends may feel bilked. These two possibilities illustrate the perpetual difficulty of recruiting financial support for high-energy physics. Orderliness certainly saves money, measured as spending per decade, but postpones discovery and encourages the dispersal of research and design teams. Too much of it would give high-energy physics grounds for fearing that their ambitions would be steadily attenuated by budget constraints. But too much competitiveness can lead to mistakes, with the consequence that the flow of funds dries up altogether.

So why not put together the hesitancy of the Congress, the opportunity of the LHC and the impending change of management at CERN to form a true international partnership for the design, construction and operation of the "next accelerator"? At the very least, the United States should apply for membership of CERN. Japan would no doubt willingly follow suit, if only to escape the unseemly (and eventually corrosive) pressure to which it has been subjected by the United States over recent months. And the world's taxpayers, so far amazingly tolerant of the demands of high-energy physics, may for a little longer be persuaded that the ambitions of high-energy physics are every bit as important and exciting as they are described. □

Unloved academics

British university teachers, already badly paid, have been done out of a pay-rise they deserved.

THE belief that academics are a gang of layabouts, not deserving of the rewards others enjoy, is deeply entrenched among British governments. Much of Mrs (as she was) Margaret Thatcher's animus against British universities and those working in them sprang from these deep wells. But the change of government has not changed attitudes. Last Thursday, the government confirmed the rumours it had started that it would not allow the salaries of academics to increase by the 7 per cent agreed between the university vice-chancellors (otherwise, 'presidents' or 'rectors') and the labour unions, but would instead insist on only 5 per cent. The vice-chancellors are right to say that the incident raises the question of who runs British universities, which are



supposedly autonomous, but which depend on the government for virtually all their income.

The truth is that British academics are so grossly underpaid that the difference between 5 and 7 per cent is probably immaterial. One consequence is that internationally mobile academics can easily be poached, another is that research people working at universities, graduate students included, cannot be decently rewarded without creating intolerable anomalies, which has the effect of constraining entry into research. Yet it is many years since annual pay settlements have matched the going inflation rate. Young people embarking on academic careers are to some degree protected by the annual increments accompanying the various academic grades, but eventually they reach a plateau that sinks slowly as inflation continues.

A university system cannot soldier on like this, decade after decade, without becoming second-rate. Good people will either go elsewhere or will be so demoralized by daily hardship that they cease to be productive. To be sure, the British government has problems: it is committed to spending much more than its likely revenue, and is embarking on a sensible campaign to spend much less. In that cause, just two weeks ago it vetoed a recommendation that senior public servants' salaries should be increased by 30 per cent, holding them instead to 5 per cent (its counter-proposal for academics). But other public servants, teachers and nurses, for example, have seen their salaries this year increase by roughly 7 per cent. Why pick on academics? Is it because the students who might have supported them have dispersed for the summer? Or does the government also believe that British academics spend all summer on some beach?

The underlying issue is indeed that of who manages the universities, which is a complicated question. On academic pay, the British government does not hide its distaste for the arrangement under which salaries for academics and support staff at universities are nationally negotiated. The system has several drawbacks; successful universities cannot easily reward those to whom success is due and, more important, struggling universities cannot corporately decide to prefer pay-cuts to extinction. The trouble is that most British universities have only insignificant resources of their own, while the arrangements for meeting their running costs from public funds are still so new (and are still changing) that no university could confidently break out of the traditional system and be sure that it would not land itself in trouble in just a few years. The paradox is that the government's actions are unlikely to speed the arrival of the goal it has in mind. □

Broadcasting science

The renewal of the BBC's charter demands the intervention of others than mere broadcasters.

THE British Broadcasting Corporation (BBC) is not an agency of the British government as, outside Britain, it is generally misunderstood to be, but an autonomous broadcasting agency whose constitution is a distinctive means of separation from

central control. The BBC's chief source of funds is a fee paid by all who own a television receiver. The fees are thus not taxes; anybody wishing not to pay can discard his receiver. The proof of the BBC's independence is that it has repeatedly been a thorn in the flesh of governments of all colours, partly because of its satire of politicians, more seriously for its critical reporting of public affairs. But now the government has a chance to turn the tables on the BBC, whose charter (including its right to licence fees) has to be renewed in 1996. Sensibly, the minister responsible, Mr David Mellor, says there will be an outline of the government's proposals in a few weeks and then a further year for debate.

The British science lobby, such as it is, must be heard from during that period. Although, when last year's Home Secretary was waging war on pit-bull terriers, a popular Member of Parliament, Mrs Edwina Currie, told a radio audience asking about the relevance of DNA fingerprinting, "I don't think dogs have DNA", the value of broadcasting (radio and television) in making science generally understandable and understood should not be understated. Despite recent improvements in newspaper coverage of science, broadcasting can make a unique contribution, and for two reasons — partly because the audience is self-selected from among those with an interest in affairs in general, and partly because broadcasting has distinctive characteristics, notably its discursiveness, its capacity to tackle often fuzzy conceptual matters and its ability to suggest that the conversation of science is also interesting and humane.

The BBC has a good record in the field. Its regular science programme called *Horizon*, now managed in partnership with WGBH in Boston, is the most obvious feather in its cap, but there is much else besides. It is true that in the recent past the BBC has been seduced into supposing that science is coextensive with natural history, partly because of the skill of Sir David Attenborough as a presenter (narrator) and of his film crews, and partly because audiences are naturally mesmerized by the sight of great animals in the wild or of multicoloured fly-catching plants swallowing their prey. But that misjudgement, not catastrophic, seems to owe something to the BBC's competition with the 'independent' or commercial television companies for audience share.

That practice, and the principle from which it stems, will be the nub of the argument in the year ahead. Should there be a public service broadcasting organization, and should it be paid for by all who receive television signals, even when they remain tuned to other channels exclusively? One of the BBC's achievements is that of marrying the didactic with the entertaining and intellectually stimulating, which is possible only within a unified editorial framework. When commercial television was introduced in Britain in the mid-1950s, holders of the right to broadcast were also required by their regulators to follow the ethos of public service broadcasting, and did so creditably, but that has now been undermined by the doctrine that the right to broadcast goes to the highest bidder. Throwing the BBC into the same pot, as some suggest, or even requiring that it should look to sponsorship for support, would spell the end of public service broadcasting when the need of it is likely to be greater than ever. □

Contradictory US trade policy opens supercomputer market to Japanese

Washington. Agreements between the United States and Japan to protect free markets for US supercomputer manufacturers are so tangled that even US officials have apparently misread them. A dispute between US agencies allowed Japanese companies to compete for two sensitive government contracts last year despite the protests of US supercomputer companies.

John Rollwagen, chairman of Cray Research Inc., told a US congressional committee earlier this month that the National Aeronautics and Space Administration (NASA) wrongly opened a supercomputer contract to Japanese companies after deciding that a 1990 agreement requires reciprocal open competition. In fact, he pointed out, the 1990 agreement is a one-way street: Japan must set a fair price for any supercomputer it wants to buy and must also take into account performance, but the United States is under no such obligation.

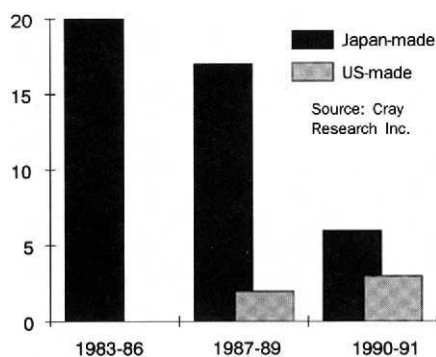
US supercomputer makers are unhappy that the Japanese are allowed to seek US government contracts when US companies have been essentially locked out of the Japanese market. The relationship between supercomputer makers and US government researchers is also a sensitive point. Cray, with the support of US government laboratories, created the supercomputer industry. Now Cray officials are concerned that their one clear advantage over their Japanese competitors — software — may disappear if Japanese companies get help in developing

software from those same laboratories.

They complain that Japanese companies have attempted to 'dump' supercomputers on the US market at implausibly low prices — even offering to donate supercomputers to the Massachusetts Institute of Tech-

Selling supercomputers in Japan

Public sector purchases
before and after 1987 and 1990 treaties



nology and a climate modelling consortium in Colorado — to gain a share of the US government and academic market. Meanwhile, Japanese companies and government laboratories have prepared specifications that require either an artificially low price or performance tailored to the strengths of Japanese machines.

In response to these complaints, the US Trade Representative (USTR) has negotiated two agreements with Japan to open the

Japanese market to US supercomputers. The first, in 1987, was generally ineffective, despite an attempt by the Japanese government to ease tensions by hurriedly buying three US machines. The 1990 agreement is considered better, but since then US companies have won only three of nine Japanese public-sector bids, despite the generally accepted technical superiority of US machines.

Given these problems, US companies were infuriated when NASA announced an open competition for new supercomputers at its Marshall Space Flight Center and Ames Research Center. In both cases, NASA had first intended to close the competitions to all but US manufacturers on the grounds of economic security.

In the case of Marshall, however, NASA was advised by another US agency that such an exemption would probably run counter to the General Agreements on Tariffs and Trade (GATT), the set of international fair trading treaties. In the Ames case, involving a more advanced supercomputer for aerodynamic simulation, NASA was able to get permission from the USTR to close the competition to non-US companies, leading a US subsidiary of the Japanese company NEC to file a formal protest. Neither competition has been concluded.

Representative John Conyers (Democrat, Michigan), chairman of the legislative and national security subcommittee of the House Government Operations Committee that held the hearings, believes that NASA was dissuaded from restricting competition by the USTR, which he says "is intent on protecting the rights of Japanese firms". Conyers says that the USTR's stance is a tactic to coax Japan to open its own markets.

Gary Edson, the USTR general counsel, denies that charge, explaining that his office had not been asked to give advice on the Marshall purchase and had approved a restricted competition for the Ames supercomputer. In his opinion, NASA decided to open the Ames competition because it was reluctant to get involved in litigation.

USTR officials say that they accept exemptions to GATT and other treaties for reasons of defence-orientated national security. But in cases where the issue is economic security, the USTR is reluctant to interfere with free trade. "Basically, we don't want our trading partners coming back and doing it to us", explains one official.

Despite protests by US companies, the Bush administration apparently takes US supercomputer makers at their word: if their machines are really better, they should be able to retain dominance of the US government market.

Christopher Anderson

Cray claims foul play in Japan

Tokyo. Days after John Rollwagen, chairman of Cray Research Inc., appeared before the US Congress (see above), his company complained that Japan's new National Institute for Fusion Science had given unfair advantage to the Japanese manufacturer NEC in its search for a supercomputer.

The protest, filed with the prime minister's office, is the first complaint to be made under a 1990 US-Japan agreement intended to give US manufacturers better access to Japan's government research laboratories. Cray's action will delay installation of a supercomputer at the fusion institute for at least three months while a committee investigates the claim. The institute is building the world's largest helical fusion device and needs a supercomputer to analyse the data (*Nature* **356**, 468; 1992).

In a press statement in Tokyo last week, Rollwagen complained that the institute's selection of NEC's SX-3 supercomputer over a Cray C90 used "odd and inconsistent measurements". He pointed out that Cray recently won a bid against the same NEC system in a contract with the US Environmental Protection Agency with a much less powerful Cray (a Y-MP system) than offered to the national fusion institute.

Atsuo Iiyoshi, director of the institute, says that the institute requires a computer with a system that can transfer huge amounts of data to external memory, a feature that favours NEC. But Rollwagen says that NEC's claims for its external optical storage device are exaggerated, and that there were other aspects of how the bids were scored that gave an unfair advantage to NEC.

D.S.

Japanese physicists say SSC will hurt domestic research

Tokyo. An overwhelming majority of Japanese high-energy physicists oppose the US Superconducting Super Collider (SSC) because of fears that their country's potential \$1.5 billion contribution to the Texas laboratory will bankrupt domestic projects. Their opposition is likely to continue the erosion of US support for the proton-antiproton accelerator, still reeling from a vote last month by one house of the US Congress to kill the \$8.25-billion laboratory.

The startling lack of support became evident earlier this year during a rancorous meeting at the High-Energy Physics Laboratory (KEK) in Tsukuba science city. The debate at Tsukuba, which is recounted in detail in the June issue of the Japanese newsletter *High Energy News*, also reveals the absence of an effective mechanism for Japanese scientists to influence government science policy.

The Tsukuba meeting, held on 28 April, was called by the High Energy Committee, a small group of high-energy physicists trying to develop a consensus on future projects. It provided a rare occasion for an open discussion of priorities and, much to the surprise of committee members, a large number of high-energy physicists took advantage of the opportunity.

The meeting was supposed to focus on

how university researchers could link up with plans by KEK to launch new domestic high-energy physics projects, including a B-factory (see below). But it quickly turned into a debate on the SSC.

One participant who opposes the SSC says that the attack was "a spontaneous revolution" after the chairman, Ycikiyo Nagashima of Osaka University, tried to direct the discussion towards the issue of university participation in domestic projects. "In the past five years there has been no discussion of the SSC problem", complained one scientist, according to *High Energy News*.

Takahiko Kondo of KEK, the leading organizer of Japanese participation in the SSC, said that "we have made efforts to provide information in SSC symposia, extended High-Energy Committee meetings, and in *High-Energy News*". But his audience rebelled when he explained that US officials have "coupled" Japan's participation in supercollider science to its contribution to the construction costs of the SSC.

"That will bring our domestic plans into crisis", complained one participant. Replied Kondo, "it's not a crisis. It [the SSC] is a salvation. The SSC will act as a bulldozer to make a breach for future plans for the high-energy community". But one opponent, speaking anonymously after the meeting,

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Yorikiyo Nagashima

says that "if it's a bulldozer, it's a bulldozer with a hell of a lot of explosives that will kill Japanese science".

Out of about 800 high-energy physicists in Japan (including graduate students) only about 100 want to participate in the SSC, says Fumihiko Takasaki of KEK, who was asked by the Ministry of Education, Science and Culture to measure the level of support for the US project. And many of its supporters are expected to retire before the SSC is scheduled to be finished in 1999. In addition, says Takasaki, no high-energy physicists at Tokyo University or Kyoto University, Japan's two leading national universities, want to participate in the SSC, meaning that it will be hard to attract top graduate students to the project.

Nevertheless, the Japanese prime minister, Kiichi Miyazawa, agreed in January to a request by the US president, George Bush, to form a joint US-Japan working group on the project. Some Japanese scientists believe that the working group exists only because debate over the SSC has been "suppressed". One young researcher at the Tsukuba meeting called for the debate to be opened to the Japanese newspapers, but his idea was quickly squelched by senior scientists.

A member of the High Energy Committee says it was considered "politically expedient" not to publicize the divisions in the high-energy community. "If we stop the SSC, the money will not reappear", he says. Furthermore, there is concern that the high-energy physics community will suffer if their opposition helps to kill the SSC.

Proponents of the SSC have used this sentiment to silence dissent. But on this occasion the amount of money involved is so huge — the United States is asking Japan for about ¥200 billion (US\$1.5 billion) — that many Japanese high-energy physicists fear for the future of their own domestic projects.

David Swinbanks

B-factory tops Japanese wish list

Tokyo. Japan's high-energy physicists may be divided in their opinion of the Superconducting Super Collider (SSC), but they are united behind what they want to build at home. Their priority is a B meson factory at the country's High-Energy Physics Laboratory (KEK), followed by a more ambitious proposal to build a Japan Linear Collider (JLC). But the fate of the JLC is very much dependent on the SSC.

The B-factory has emerged as the favoured next project because its cost — ¥40 billion (US\$320 million) — can be fitted into the resources available to KEK. In addition, now that the US government has delayed consideration of proposals from the Stanford Linear Accelerator Center and Cornell University until at least 1997, Japan has a chance to be first.

Japan's B-factory would be built within the ring of KEK's present TRISTAN electron-positron collider. The idea is to build an electron-positron collider with a very high luminosity — about 100 times that of TRISTAN — to produce B mesons by the truckload. These mesons, which are composed of bottom quarks and antiquarks, will be used to test quark-mixing theories and the physics of the strong interaction force. The Ministry of Education, Science and Culture has already assigned a small research and development budget to KEK. Japan's high-energy physicists believe that the education ministry will eventually ask for money to build a B-factory, but not until the conclusion of talks between the United States and Japan on the SSC — certainly no sooner than the end of the year.

The future of the JLC is less certain. Researchers at KEK and Tokyo University want to build two linear colliders (linacs), each about ten kilometres long, that would collide electrons and positrons at very high energies. Initial plans called for two 500-GeV linacs to yield collisions at 1 TeV (see *Nature* **344**, 8; 1990). Recently, however, JLC's proponents have reduced the scope of the collider to 150 GeV to save money and to simplify the technical challenges.

D.S.

German astronomers fight rumoured closing of institute

Munich. Theoretical astrophysicists at the Max Planck Institute for Astrophysics at Garching near Munich say that their institute is on the brink of being closed — for all the wrong reasons.

The researchers accuse their funding agency, the Max Planck Society, of using a prolonged search for a new director as an excuse to shut down the institute, saving money into the bargain. They say that their



Will Max Planck pull the plug on its astrophysics institute?

scientific productivity and role in training students are being ignored.

The society says that the institute's fate will not be decided until October, when it receives the recommendations of two committees — one looking for a successor to Rudolf Kippenhahn, the institute's long-time director who retired last year, and the other reviewing the society's astronomy programmes. Officials insist that money is never a prime motive in closing down an institute, whatever its size, and that closure in fact offers the opportunity to start something new. In the meantime, scientists from around the world have rallied to the institute's defence.

The controversy arose after the society formed a committee, composed of nine Max Planck scientists and an international group of six independent scientists, to recruit a new director. While researchers wanted to abandon their single directorship in favour of the standard Max Planck system of a board of around five directors, the committee persisted in seeking — so far unsuccessfully — just one director. When one candidate turned down the job, the committee considered this as an indication that the institute may not be strong enough to attract a top-quality administrator.

The 31 scientists at the institute fear that the society's senate will use this as a reason to justify closing the institute. And they fear the worst after acting director Wolfgang Hillebrandt was asked recently to stop looking for candidates.

But Peter Schneider, a researcher at the

institute, says that the society may lose more science than it bargained for. And the savings will be small, he says, pointing out that the institute's budget represents only 0.7 per cent of the society's annual budget of DM1.4 billion (US\$900 million).

The society, which supports 56 large-scale research institutes in Germany, is certainly looking for ways to save money. Its budget has effectively stagnated in the last 20 years, and since reunification it has adopted several East German science programmes. The society continually assesses its programmes, shutting down parts that are no longer productive; in the past 10 years, 15 departments and three research groups have been replaced in this way. Two institutes were also closed, although both were reopened under different names and with the same staff.

By law, anyone working for more than 15 years at a Max Planck institute cannot be laid off. Those not offered jobs in other departments or institutes are eligible to receive a proportion of their salaries for a time, based on length of service.

At the moment, a commission of six scientists is reviewing the status of astronomy research, which is carried out in four institutes and one department. In the autumn, this commission will report to Hans Zacher, president of the society, who will combine its report with information from the recruitment committee to decide the future of the astrophysics institute.

One possibility is a merger of groups from the theoretical institute into other experimental institutes. But Schneider says that this would impinge on the freedom of his colleagues to choose their own projects. Such a move would also sacrifice the advantage of being near Munich, home of several large physics institutes and the European Southern Observatory.

Jean Audouze, current president of the institute's visiting committee — an international group of scientists who meet every two years to discuss the scientific progress of a particular Max Planck group — has received letters of support from scientists in several other countries who fear the institute may close. Each year the institute receives scores of visiting scientists, who collaborate for short periods of weeks or months; at present it is also host to 35 students.

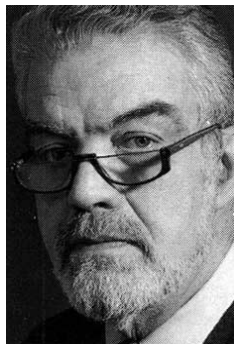
Audouze, president of the Paris Institute of Astrophysics, says that not a single scientific argument could support closure. "The institute does excellent research, and all who visit recognize that its strengths are at all levels — senior, junior and students", he says.

Alison Abbott

EMBL director quits after long fight over expansion

Munich. The director of the European Molecular Biology Laboratory (EMBL), Lennart Philipson, has resigned, saying that he is tired of a system that allows a single country to veto a project that has been approved by science committees. Delegates from each of EMBL's 15 member countries had long been aware of Philipson's frustration in trying to expand EMBL, but none were prepared for his announcement at this month's council meeting.

EMBL, set up in 1974 in Heidelberg, Germany, has grown considerably in the last decade under the leadership of Philipson —



Lennart Philipson

its staff from 300 to almost 800, and its annual budget from DM42 million (US\$27.5 million) to DM80 million. That growth has been matched by performance: EMBL now ranks as the world's third most influential molecular biology institute, according to citation rates per paper compiled by the Institute for Scientific Information (ISI) in Philadelphia.

Philipson had a grand vision for a European venture that had little room for the 'nationalist stance' of some member countries and their reluctance to take money for their own national programmes. This attitude has caused considerable friction within the council.

He is also angry that council delegates have no negotiating power but rather attend meetings only to convey the decision of their governments. Last year (see *Nature* 351, 91; 1991), he criticized those who shape decisions about European collaborative projects, describing them as either "failed in research" or "inappropriately trained".

But many EMBL scientists also doubt the wisdom of a continuing expansionist policy. The sense of the laboratory as a huge extended family has disappeared, and a policy that prohibits researchers from staying longer than nine years makes difficult contact between projects.

Philipson will not be leaving until next April, two years before the end of his contract, but the search for a replacement may be difficult. Philipson succeeded EMBL's first director, Sir John Kendrew, in 1982 and was reappointed in 1989 after EMBL was unable to find a scientist willing to leave the bench temporarily to become an administrator.

Alison Abbott

UK agricultural funding leans towards policy

London. Agricultural scientists are concerned that changes in the way the Ministry of Agriculture, Fisheries and Food (MAFF) commissions research will damage their work.

Starting in 1994, MAFF will spend 5 per cent of its budget on projects of its own choosing, selected through competitive bids, rather than waiting for researchers to submit their ideas and picking the best ones. As one academic commented, it is like saying to the researcher, "if you are going to have a bright idea, this is the one you ought to have". In a current annual budget of £137.3 million (US\$260 million), the new approach would receive £7 million. MAFF hopes to increase that amount if all goes well.

MAFF also wants to make the organizations it funds more responsive to its needs by shortening the time required to notify them of changes in the nature or scope of their funding. It now informs recipients a year in advance of the end of a grant and six months in advance if new work is to be commissioned.

MAFF research contracts are the principal source of funding for some Agricultural and Food Research Council (AFRC) institutes and research associations. In many cases, the work they do is not duplicated elsewhere, and there is insufficient expertise to take up research work that is lost.

The council will be getting £1.5 million less from the ministry beginning next April in the first of a series of annual budget cuts. MAFF currently spends £34 million with the AFRC. While the AFRC is confident that its researchers will win back a large proportion of the money through competitive bids, institutes fear that the new approach will mean an uncertain future for many scientists.

"Competitive tendering may be an exercise we can only do once", says one AFRC researcher. "Many of these people will not

be there in 12 months' time to tender again." The impact of the change will be felt even on those groups not receiving contracts from MAFF. "We try very hard to link work that is funded by different sources", says the AFRC scientist, "but the [new system] will break up the continuity."

MAFF's quest for greater flexibility reflects the growing importance of policy considerations in setting out the research agenda. Over the last few years, budgetary responsibilities for research and development have largely been transferred to policy groups, with scientists as advisers. Priorities have changed accordingly; for instance, the emphasis in food research has switched from the underlying technology to safety and nutrition.

Changes in priority are particularly damaging when they occur on short notice. The explosion of interest in bovine spongiform encephalopathy (BSE), or mad cow disease, deprived other related areas of both funds and personnel. Some researchers fear that BSE teams could be hurt when the emphasis changes again. In the meantime, postings tend to be short-term and the people involved complain of being picked up and dropped according to the fashion, without regard for the quality of their work.

"The final line does not seem to relate to the quality of the science", says one senior AFRC researcher. "It's not a science-driven process any more."

The emerging structure favours agricultural organizations such as the research associations and Horticulture Research International, an independent body formed two years ago from an AFRC institute and the horticultural units of MAFF. They are organized along more commercial lines and operate fewer long-term 'public good' research programmes, allowing them to respond faster to customer demands.

Ian Mundell

Germany links biology centres

Munich. Germany has taken its first step towards improving its biomedical research by linking five large research centres. These include the large cancer research centre in Heidelberg and the newly saved Max-Delbrück Centre for Molecular Medicine in Berlin-Buch, eastern Germany.

The network of institutes will share committees to oversee appointments and to evaluate the research done in each unit. Evaluation committees will be international, and research activities in the institutes will be loosely coordinated.

The German Ministry of Research, the BMFT, which earlier this year adopted the recommendations of its advisory committee on biological research (see *Nature* 357, 182; 1992), has donated DM4 million (US\$2.6 million) to get the project going. Harald zur Hausen, director of the Heidelberg centre, hopes that this marks the start of a more extensive national cooperation involving other research institutes. But university departments are expected to resist the idea because of their concern about a loss of independence.

Alison Abbott

MITI prepares to fund cold fusion by another name

Tokyo. At a time when few scientists believe in cold fusion, Japan's powerful Ministry of International Trade and Industry (MITI) is ready to spend millions of dollars on the phenomenon that grabbed headlines worldwide in 1989.

Last week, the ministry's Natural Resources and Energy Agency confirmed newspaper reports that it is hoping to start a government-industry project in the next fiscal year (beginning 1 April 1993) to pursue possible application of cold-fusion experiments in the energy industry. It would be the first significant government funding of cold-fusion research in Japan. MITI's move follows recent claims by Akito Takahashi of Osaka University of excess heat generated by passing an electric current through palladium electrodes immersed in heavy water — much as chemists Stanley Pons and Martin Fleischmann reported in 1989.

Tomihiko Taniguchi, director of the agency's Electric Power Technology Division, says that MITI is not claiming that Takahashi's experiments involve fusion, preferring to call the phenomenon "hydrogen energy". MITI's reluctance to use the words 'cold fusion' stem from scepticism of the phenomenon and its desire not to appear to be encroaching upon the research territory of other ministries.

Most Japanese scientists abandoned cold-fusion research after an initial burst of enthusiasm in 1989. But a small number of die-hard enthusiasts in the universities have continued experiments using the small annual grants of a few million yen (US\$1,000–\$2,000) that university departments receive from the Ministry of Education, Science and Culture. Taniguchi says the project must still be discussed internally before the ministry submits a budget request at the end of next month. But he expects it to be "hundreds rather than tens of millions of yen" to support a laboratory with researchers from academic institutions and industry.

Electric utility companies and material processing industries are interested, Taniguchi says. MITI would like to persuade the "rather conservative" energy industry to back this "high-risk basic research", he says, because of its potential for a "big payoff".

Japan's leading 'hot' fusion researchers are adopting a wait-and-see attitude. Although they are not yet convinced that the ministry will in fact spend the money on cold fusion, they seem prepared to tolerate the initiative if no claims of fusion are made. As one leader of fusion research puts it, "cold fusion is not science".

David Swinbanks

Fusion reactor finds delay is price of global cooperation

Washington. International cooperation is supposed to be the salvation of big science, but a four-country effort to design and build the next-generation fusion test reactor shows that the outlook for such collaborations is murky as well. This week, 18 months behind schedule, the United States, European Communities (EC), Russian Federation and Japan were expected to sign an agreement for a six-year, \$1-billion engineering design phase of the International Thermonuclear Experimental Reactor (ITER).

The pact, signed in Washington, will permit the three design centres, located in San Diego, California; Garching, Germany; and Naka, Japan to begin hiring what are expected to be as many as 180 scientists and engineers and 120 support staff. The fact that three centres were required, instead of the one venue first envisioned, suggests that even bigger problems loom when the partners try to decide where to build the actual reactor. Paul-Henri Rebut, the project's director-designate, wants to settle on a site in four years. Even then, the reactor could not operate until 2005.

The partners had expected the engineering phase to flow relatively quickly from an earlier and smaller conceptual design completed in late 1990. Although an agreement was reached in principle last August — eight months behind schedule — it has taken almost a year to gain the required approval of the member governments.

ITER's official cost is \$5.8 billion, although its supporters admit that the eventual price may be close to the \$8.25-billion budgeted for the Superconducting Super Collider. US officials are fond of citing that giant particle accelerator, under construction in Texas, as big science conceived and carried out the wrong way, without full international participation.

Great care has been taken to ensure that the three design centres are seen as coequals, although Rebut, now head of the EC's Joint European Torus (JET) project in Culham, England, will work in San Diego. The project's oversight and design integration functions will also be there, although they may rotate as part of a continuing effort to keep the sites equal.

The Garching site, outside Munich, will be responsible for designing the reactor vessel and its contents. That group will be headed by Ronald Parker of the Massachusetts Institute of Technology. The Japanese centre, led by Michel Huguet, also from JET, will design components outside the vessel, including the five-storey-high superconducting magnets that will shape and confine the fusion plasma within the reactor. Yasuo Shimonura of Japan's Atomic

Energy Research Institute will be the project's third deputy director, overseeing design integration from San Diego.

Parker says that he and other scientists have been frustrated by the delays, and that it has been difficult to sustain the project's momentum in the absence of a formal agreement. Ikuo Makino, until last month the director of the technology development division of the Atomic Energy Bureau in Japan's Science and Technology Agency, blames the delay on "cultural" problems among the partners. Although US and European officials enjoy 'hard' negotiations, he says, Japanese shrink from them and the Russians fall in the middle. But he admits that negotiations will become more difficult as the project advances.

With regard to selecting a site, Parker suggests that the reactor may be less of a prize than it appears. The host country will probably pay twice as much as its partners because it will bear the bulk of conventional convention costs. With the United States increasingly sceptical of any large science project and the Russians too poor to afford one, "our nightmare is that no one will want to host it", he says.

Parker has little doubt that the reactor, which will produce a minimum of 1,000 megawatts of fusion power for at least 1,000 seconds, is technically feasible. "The joke around here is that all these machines look the same in an eight-and-a-half by 11 [inch] viewgraph", he says. "It's just the man standing next to it who looks smaller."

Dave Kramer

Hoyle statue unveiled

London. Many hatchets were elegantly buried last Saturday (18 July) when a bronze statue of Sir Fred Hoyle was unveiled on the grounds of the Institute of Astronomy at the University of Cambridge. Hoyle, from the outset of his academic career a fellow of St Johns College, founded the Institute of Theoretical Astronomy (as it was then called) in the early 1960s. He resigned his university positions less than a decade later, feeling slighted by the university.

At the unveiling, Hoyle's erstwhile collaborators, Thomas Gold, Hermann Bondi and R.A. Lyttleton, lauded his contributions to astrophysics and cosmology. Hoyle said that the site is peopled by ghosts including that of Eddington, who had plotted the 1919 solar eclipse to within 100 yards, and wistfully wondered whether his own ghost might compare itself with that of others seeking an elusive goal and failing to find it.

John Maddox

Burris appointed to head Woods Hole marine laboratory

Washington. The Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, last week abandoned a century-old tradition of electing a working scientist to head the institute by choosing a scientist-turned-administrator skilled at raising funds and building consensus.

The choice of John Burris, a 43-year-old marine biologist who has spent the past



John Burris

eight years at the National Academy of Sciences, reflects the problems facing small independent research institutions. MBL draws the bulk of its financial support from federal grants won by its few year-round researchers, but competition for such grants has

grown so fierce that the venerable laboratory, with its small endowment, is struggling to preserve its status as one of the pre-eminent summer research centres. Burris replaces Harlyn Halvorson, who has reached the mandatory retirement age.

Despite the laboratory's difficulties, some members of the search committee wanted to fill the slot with a working scientist. MBL has "always had a practising scientist" as a director, says Roger Sloboda, a cell biologist at Dartmouth College and an MBL trustee. "People assumed that was a given."

But the committee soon realized that a working researcher would be unable to oversee an organization as large and troubled as MBL. So after a year-long search, the committee unanimously selected Burris, who holds a PhD from the Scripps Institution of Oceanography in La Jolla, California. Burris has spent the last eight years as an administrator with the academy's National Research Council, most recently as executive director of its Commission on Life Sciences.

Burris, who takes office as director and chief executive officer in September, says he welcomes the chance to revitalize MBL. "I think it's an exciting position, a challenging position", he says.

Those in the MBL community who know his credentials have faith that Burris can meet the challenge. "MBL is very much in need of astute management", says John Dowling, a neurobiologist at Harvard College who has spent the past 24 summers at the laboratory, "and that's exactly what he's going to provide."

Traci Watson

Problems delay emergence of Moscow research centre

Moscow. Glenn Schweitzer does not have a permanent office. He cannot afford the cost of a modern peer-review system. And he is executive director of an institution, the International Research and Technology Centre in Moscow, that does not yet exist.

But Schweitzer, on a two-year leave of absence from the US National Academy of Sciences, has access to something that hundreds of Russian scientists from the former Soviet defence establishment would like a share of: \$70 million pledged by industrial nations to help them transfer their skills to the civilian sector.

The centre that presidents Boris Yeltsin of Russia and George Bush of the United States agreed to create six months ago is expected to open by the autumn. Its purpose is to support Soviet scientists previously engaged in research on weapons of mass destruction, including chemical and bacteriological as well as nuclear weapons. The final obstacles are technical ones relating to the translation of documents, says Schweitzer, and Russian academician Eugene Velikhov says he sees "no reason for further delay".

The centre will not be a laboratory in its own right, but rather a clearinghouse to match worthy ideas with sources willing to pay for them. Most of the work is expected to be in weapons disarmament, environmental cleanup, energy development and conservation and the transition to a market economy, although some will focus on fundamental research.

Proposals will be reviewed first by scientists from around the world to decide their technical merit, then judged by a board of science administrators from each of the contributing countries. Individual proposals will then be paired with sources of funding, both public and private. Russia will have the right to reject any project.

The first recipients are expected to be scientists from the formerly secret military research centres at Arzamas and Chelyabinsk. Negotiations are also under way with Ukrainian specialists in ballistic missiles and, in Kazakhstan, with specialists from the town of Kurchatov (near the Semipalatinsk proving ground) engaged in nuclear testing. A sister centre is being set up in Kiev, for Ukrainian weapons scientists, with \$12 million in Western funding.

Schweitzer arrived in Moscow last month,

hoping to occupy space in a science park being built at Moscow State University. But work on the new park is progressing more slowly than planned, forcing Russian authorities to continue their search for a permanent home in Moscow for a staff that is expected to number two dozen before the end of the year.

The original plan was for the centre to be at Troitsk, a small town near Moscow well equipped with communications, hotels and even schools for the children of foreign staff. But Russian military administrators, attracted to the centre by a decision that half of their salary will be paid in hard currency, apparently wanted a site closer to home.

Another logistical hurdle is the difficulty of conducting a rigorous peer review of the proposals that are expected to pour into the centre. "Communications links are still a major problem", Schweitzer says. E-mail cannot accommodate all the graphic elements of a typical proposals, and "faxing to countries around the world is

prohibitively expensive".

Even more troubling is that few of the Russian scientists, having worked in secrecy, are known to their international colleagues. "We should get good reviews of the potential payoff and technical merits of the proposal", says Schweitzer, "but it'll be hard for reviewers to assess the technical skills of the people involved if nobody knows their work". One safeguard is careful monitoring of work in progress by staff at the centre, as well as by Western partners.

Not everybody shares the enthusiasm of the founders for the new centre and their confidence in the ability of government bodies to adapt to new conditions. George Soros, who has promised to supplement the salaries of researchers working through the centre by \$100 a month (see *Nature* 357, 431; 1992), says that "US bureaucrats are better at turning other people's bucks into their own limousines". And Maxim Frank-Kamenetskii, a prominent researcher at the Institute of Molecular Genetics of the Russian Academy of Sciences, believes the venture "is doomed to fail" because the historically high barriers to the flow of information within the former Soviet Union will prevent people from being able to judge their colleagues' work accurately.

Vladimir Pokrovsky

Anything left for civilian science?

Washington. The United States has pledged \$35 million to two research centres in Moscow and Kiev intended to give former Soviet weapons scientists the chance to make peace, not war. But getting anything close to that amount for civilian scientists, who need support just as badly as their military counterparts, has proved much more difficult.



D. Allan Bromley

D. Allan Bromley, science adviser to President George Bush, has been trying to persuade the US State Department and the Agency for International Development (AID) to reallocate some of AID's current budget, in the fiscal year ending 30 September, for direct aid to scientists in the former Soviet Union. AID is spending \$235 million this year to help Russia dismantle and safeguard its nuclear arsenal, including \$150 million in new money from a supplementary appropriation passed earlier this year.

AID has given \$2 million to the National Academy of Sciences to pay for as many as 150 Russians to come to the United States and work temporarily with US researchers. Bromley wants another \$25 million for the National Science Foundation (NSF) and the National Institutes of Health (NIH), the two federal agencies in the best position to coordinate

such a programme of small grants. NIH has spent about \$3 million this year on several programmes to help those in the former Soviet Union, the most popular being long-term visits to NIH laboratories and supplementary grants to US scientists working with Russian scientists. NSF has spent slightly more than \$2 million this year on similar cooperative research projects. The agencies raised the money by taxing other programmes.

But Bromley's plea, taken from a recommendation made by experts assembled by the National Academy of Sciences, has apparently fallen on deaf ears within the Bush administration. "He struck out", says one NSF official. An NIH administrator is a bit kinder. "So far that idea has not borne fruit", he says. "It would be pretty hard to do anything like that this late in the fiscal year."

But Bromley has not given up hope. Civilian scientists in the former Soviet Union "have an immediate need" for US funding, he says, and "we don't want to wait for complex mechanisms that will take years to work out. I'm still trying to get them money now, in FY [fiscal year] 92 dollars."

Jeffrey Mervis

Correction

A photograph in last week's issue of *Nature* (358, 180; 1992) identified as being of D. Allan Bromley, US presidential science adviser, was in fact a picture of John Rollwagen, chairman of Cray Research Inc.

What to believe of science

SIR — *Nature* has exposed two enemies of the eternal verities, George Walden and Bryan Appleyard (356, 729; 1992). I can do no better than to quote from the graduation address of 22-year-old Herbert M. Evans in Berkeley, 1904.

Why not then limit scientific and in particular biological research to problems sure of a practical outcome? Were not such a question sometimes seriously asked there would be no need of answering it.

Nothing in all our evolution toward civilized life is now so well established as the fact that pure research must proceed, and is the mother of applied science. Man must know natural laws widely and generally, before we can harness them for human use. To fail to recognize the scientific fountainhead of our information would be to limit forever the conceptions of the human mind, to draw around it a fixed horizon. . . .

Through a purely theoretical interest in the structure of the sugar molecule, the great Pasteur was led to the brilliant investigations of fermentation which had so great a practical bearing on the industries of France.

So it has been in every science. . . .

Thomas H. Jukes

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SIR — Does science leave room for soul? Yes, of course it does and only those with a most superficial reading of the bangs-and-stinks-and-big-machines could think otherwise. One 'purpose' of science is to allow us to see and to understand the underlying beauty of the organization of reality. It is only those who have not seen or who cannot understand this beauty who think science soulless, or who insist on calling the Universe a machine; if it is, it is a soft machine.

If Appleyard asks for a common belief in something, why not a common belief in reality? I know that reality, often being both stranger and more interesting, can be harder to believe in than your common-or-garden miracle, but can belief in an intangible be counted purposeful? What if there is a supreme being whom I choose to believe in? Has it or anyone's belief in it relieved, for example, the suffering Bosnians? I may as well believe in fairies.

And finally, to social violence and damnation. It takes a truly perverse, if politically quotable, reading of world history to cite today as an especially socially violent time. Have not many of the most horrific examples of social violence coincided precisely with the highest 'social awareness' of eternal damnation? Bless the Crusaders, the Conquistadors, the Germans of the Nazi era, the church-going slave-traders, not to men-

tion the Spanish Inquisition. The religious, purposeful, soulful mechanics of untold abominable deaths. Believers.

Let science then erode such belief.

Simon L. Goodman

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SIR — It is amazing to read in *Nature* a virulent attack on Appleyard's book seen as a reinforcement of belief in opposition to science, and, two pages later, to find the word "doctrine" applied to the Big Bang hypothesis. Is science presenting itself as a new religion, adopting the appropriate vocabulary? This example can only comfort those who, like Appleyard, think so.

J. M. Gillis

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Marine explosions

SIR — Your report on the postponement of a marine explosion planned to have been carried out in Cardigan Bay by the British Geological Survey (BGS, *Nature* 357, 183; 1992) does not, unfortunately, refer to the Agreement on the Conservation of Small Cetaceans of the Baltic and North Seas, which Britain has recently

signed. The remarks attributed to me as a member of the Sea Mammal Research Unit should have been attributed to the interim secretariat for the agreement, which will be housed at this unit under my direction.

The agreement requires signatories to "work towards . . . the prevention of . . . significant disturbance (to small cetaceans), especially of an acoustic nature". Thus it would be unacceptable for organizations in Britain, Germany or Sweden (all of which have signed the agreement) to let off underwater charges without following some guidelines to minimize their impact on small cetaceans. The BGS had developed such a set of guidelines which was, I believe, within the spirit of the agreement.

This case has brought the potential effect of underwater seismic experiments on dolphins and porpoises to the public's attention. However, as your reporter points out, many other, much larger, detonations are carried out each year. I would encourage any organization contemplating such activity to contact the interim secretariat for the agreement for information on the potential risks to small cetaceans and how these may be reduced.

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Fact and fiction in alignment

SIR — We have discovered a startling similarity between a dinosaur DNA sequence reported in the novel *Jurassic Park*¹ and a partial human brain cDNA

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HUMXT 317  GCGTTGCTGGCGTTTTCATAGGCTCCGACGCCCTGACGAGCATCACAAAATCGAGCTCAA
DINO1  1  GCGTTGCTGGCGTTTTCATAGGCTCCGACGCCCTGACGAGCATCACAAAATCGAGCTCAA
DINO1  670  GCGTTGCTGGCGTTTTCATAGGCTCCGACGCCCTGACGAGCATCACAAAATCGAGCTCAA
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HUMXT 234  GTCANAGGTGGCGGAACCCGACAGGACTATAAAGATACCGAGCGTTTCCCGCTGGAGCTTCC
DINO1  61  -----GTTGGCG- AAACCCGACAGGACTATAAAGATACCGAGCGTTTCCCGCTGGAGCTTCC
DINO1  730  -----GTTGGCG- AAACCCGACAGGACTATAAAGATACCGAGCGTTTCCCGCTGGAGCTTCC
```

sequence from the Venter laboratory described in *Nature*² (see figure). The dinosaur sequence (Dino1) consists of duplication, with 117 base pairs from the first member of the repeat aligning with the human sequence, HUMXT01431, at the 95 per cent level of identity with only two gaps. The extraordinary degree of nucleotide sequence conservation between organisms as distantly related as dinosaur and human suggests strongly conserved function. Expression of HUMXT01431 in human brain raises the possibility that the dinosaurs were smarter than has been supposed, arguing against the hypothesis that their extinction resulted from lack of intelligence.

Our discovery also seems to raise the interesting legal question as to whether the copyright on *Jurassic Park* takes precedence over the pending patent on the human sequence. However, it appears that neither group is entitled to legal protection for its sequence, because both sequences also align with cloning vector pBR322, raising the possibility that both groups inadvertently sequenced vector DNA.

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1. Crichton, M. *Jurassic Park*, 102 (Ballantine, New York, 1990).

2. Adams, M. D. et al. *Nature* 355, 632–634 (1992).

Public opinion on gene patents

SIR — In connection with the two patent applications by the US National Institutes of Health for numerous human genes (*Nature* 354, 171-2, 174; 1991; 355, 103, 104, 292; 1992), there are ethical arguments that the human genome is the common property of all human beings, and no one should be able to patent mere sequences of it. These include the principle of distributive justice and the shared possession of the sequence by all members of the human race¹.

(also conducted using mail response, except for the public sample which was obtained by face-to-face interviews)⁴.

Especially at the time when policy is being decided, international public opinion studies should be conducted to find eligibility criteria for patenting genetic material which are more acceptable to public, scientists and industry worldwide. In light of the results of this survey, it is clear that people (including

hope of stable convergence in the vicinity of the truth^{5,6}.

It is interesting to note that there is a perfect analogue to Occam's razor in the biological world of evolution; the pressure to optimize metabolic efficiency. Given a new catalytic 'problem' to solve within a cell, a very large number of candidate 'solutions' will arise via mutation. They will involve polypeptides of varying lengths and synthetic pathways of varying complexity, just as for a given set of observational data there will be an ensemble of models of varying complexity that 'fit the data'. In the biological world, natural selection will, in the long run, favour the metabolically less expensive solution — usually the shortest polypeptide that can do the job — just as Occam's razor requires that preference be given to the simplest hypothesis that 'fits the data'. This will come as no surprise to the Scots, who will recognize the general principle as simple frugality.

Allan Goddard Lindh

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PUBLIC OPINION OVER PATENTING IN JAPAN AND NEW ZEALAND

	Public		Scientists		High-school biology teachers	
Country:	NZ	Japan	NZ	Japan	NZ	Japan
Sample:	2,034	470	258	479	277	227
Subject matter						
New inventions	93	91	95	94	88	92
Books, information	85	73	81	82	72	77
New plant varieties	71	60	66	78	49	61
New animal varieties	59	49	63	74	51	60
Genetic material from plants/animals	51	37	53	46	34	38
Genetic material from humans	—	29	—	35	—	29

The knowledge that is applied to obtaining a DNA sequence is the result of the efforts of numerous past and present scientists throughout the world and the research involved in developing the various techniques was paid for by people of many countries. Legal principles consistent with the underlying ethical arguments are to be found in article 27 of the United Nations Declaration of Human Rights, that has been signed by most countries of the world². There are two 'rights' that everyone should share in: (1) "in scientific advancement and its benefits", and (2) "protection of the moral and material interests resulting from any scientific, literary or artistic production of which he is an author". One can debate whether possession of a DNA sequence makes a person an author of it (albeit, a joint author), but we cannot call a DNA mapper or sequencer an author.

Most countries will exclude matter from being patentable if it offends "public morality". A randomly selected nationwide mail response opinion survey was conducted in Japan in late 1991 among different groups of the population³. Included was the question "In your opinion, for which of the following should people be able to obtain patents and copyright?" The subject matter as shown in the table was listed with three responses, "approve", "disapprove", "don't know". The results (see table) are compared with those of a May 1990 survey in New Zealand

scientists) may not agree with the patenting of human genetic material.

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2. Sieghart, P. *The Lawful Rights of Mankind* (Oxford University Press, Oxford, 1985).
3. Macer, D. *Attitudes to Genetic Engineering: Japanese and International Comparisons* (Eubios Ethics Institute, Christchurch, 1992).
4. Couchman, P.K. & Fink-Jensen, K. *Public Attitudes to Genetic Engineering in New Zealand* (DSIR, Christchurch, 1990).

Natural selection

SIR — Over the past few decades, Sir Karl Popper's work at the interface between philosophy and biology has given birth to a small school of philosophy that emphasizes the continuity of information structures across the biological and cultural realms¹. This effort grew out of the recognition of the similarities between mutation and natural selection in biology, trial and error in learning, and conjecture and refutation in science^{2,3}. In science, however, parsimony (improbability in Popper's terminology⁴) is needed along with hypothesis, deduction and experimental test. William of Occam's dictum that "What can be done with fewer assumptions is done in vain with more" must be heeded if one wishes to have a scientific process with any

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4. Popper, K. R. *Conjectures and Refutations* (Harper and Row, New York, 1963).
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6. Jeffreys, W. H. & Berger, J. O. *Am. Scient.* 80, 64-72 (1992).

Mistaken view

SIR — Daniel N. Robinson (*Nature* 357, 187; 1992) misstates the view of National Institutes of Health (NIH) scientists in his gratuitous commentary on our ethical sensibilities. Contrary to his claims, I know of no one who expressed "surprise" or objection to the "speed with which [NIH Director] Bernadine Healy ordered an inquiry" into allegations of animal abuse. On the contrary, the charges raised serious ethical issues and required investigation. Many NIH scientists did, however, object to the fact that uncorroborated allegations precipitated the hasty suspension of approved research projects.

An NIH investigation eventually discredited the charges, showing that the "catalogue of horrors" cited by Robinson was imaginary.

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Between Stockholm and Rio

Lord Zuckerman

The first international conference on the global environment was held in 1972. Has the world come any closer to solving its environmental problems in the intervening 20 years?

WELL before the formal opening of the Rio conference, it was clear that it was not going to produce a key to the Utopia that had been dreamt up in the minds of thousands of friends of the Earth after the corresponding meeting in Stockholm in 1972. The climate there had been gloomy with fears that the rate of growth in food production would be unable to keep pace with that of the world's population; that as industry increased its output to meet ever-increasing need and demand, the result would be more pollution of the air we breathe, of the water we drink and of the earth we tread; that the effort to match a world-wide demand for higher standards of living would mean that the natural resources on which we depend would dry up; that as a result of human predation, irreparable damage would continue to be done to what is now called the 'biodiversity' of our planet. As the title of a book that was much publicized at the time put it, there are 'limits to growth'. This book had little, if indeed any, academic merit, but its title has never ceased to resound.

Since that first meeting in 1972 the environmental equation has gone on increasing in complexity. Stockholm barely touched on the problem of global warming, nor was the issue of population dealt with in any depth (it also failed to be at Rio), nor do I recall that 'ozone' entered into any of its discussions. In the years between the two meetings these and all manner of other environmental worries have engaged the attention of governments and of a host of non-governmental organizations. More and more people have been made aware of the fact that the Earth is not only limited in size, but that any one country's problems often affect, either directly or indirectly, those of others.

Nonetheless, the Rio conference took off in a far less pessimistic mood than had Stockholm. The hype about one country's carbon dioxide emissions affecting the greenhouse in which we all live had inspired the hope that governments were ready to reach a mandatory international agreement to have the levels stabilized or even reduced by a given date. It was a hope that was totally unjustified. Weeks before the opening of the meeting, the president of the United States, the country accused of being responsible for 25 per cent of all the CO₂ put into the atmosphere, had made it

plain that he would not sign a CO₂ emission reduction agreement that set a fixed date for its implementation. Only a few days before the meeting was due to begin, Carlo Ripa di Meana, the European Commissioner for the Environment — having failed to get the members of the European Communities to agree to a tax on CO₂ emissions — declared that he would not attend. He had "always fought for an environmental policy based on facts, obligations, constraints and

declaration that endorsed the 1987 'recommendation' of the UN Commission headed by Madame Brundtland of Norway that the world's economic growth should be "sustainable", and that the needs of the present generation should be satisfied without compromising those of future generations. A declaration of 'principles' for the achievement of the sustainable growth objective, with a voluminous document called *Agenda 21* as a guide to action, was also approved, as was the setting up of a new UN Sustainable Development Commission. Rio, as Baroness Chalker, speaking for the British Government, put it, set an "Agenda for the 21st Century and beyond"¹. Whether the agenda results in any truly significant action will be for future generations to decide.

Like Stockholm, the significant lessons that Rio has left behind are that national interests differ; that there is a difference between national and global environmental problems; that long-term and short-term social and environmental issues do not belong in the same category; and that development in the poorer countries almost inevitably brings in its train not only financial and managerial, but also new environmental problems. Another major lesson is that, in practice, the solutions to most environmental problems lie almost entirely in the political and economic domains. The blame for the sense of let-down that Rio has left behind can be shared between those who for political reasons had made the world expect too much from it, and the over-enthusiastic environmentalists who had proclaimed that the meeting would be the most important ever to be convened.

Because he realized that divergent national interests would mean that many important issues would be either avoided or treated in a partisan way, Maurice Strong — the Canadian who was secretary general of both meetings — had recruited Barbara Ward, a well-known economist, and René Dubos, a prominent medical scientist, to prepare a general agenda or 'conceptual framework' as background for the earlier Stockholm conference. The result, a book entitled *Only One Earth*², is as relevant today as it was when it appeared 20 years ago, provided that it is read in parallel with the World Bank's development report for 1992, issued just before Rio³.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Gloomier days — Indira Gandhi at Stockholm.

precise commitments, and not on pretty words".

The draft agreement to stop the destruction of the habitats of rare creatures and plants had also been watered down to a voluntary undertaking, which President George Bush again refused to sign because, for one thing, it implied the automatic transfer to developing countries of "intellectual property" of potential industrial value. Other dreams of firm and realistic agreements, as opposed to signatures to vague and unenforceable conventions, also came to nothing. The fact is that the more the problems over which vocal environmentalists would have their banner fly, the greater the hopes they raise, and the more certain is the subsequent disappointment.

Rio will be remembered mainly because of the media attention that it received, and because it had attracted to one meeting more than 100 heads of state and representatives of some 50 others. But in effect it ended mainly in a

The final paragraph of the opening chapter of Ward and Dubos's book crystallizes the basis of all environmental problems. The biosphere of man's inheritance and the technosphere of his creation are out of balance, "indeed, potentially in deep conflict". Industrialization produces wealth but also enormous social inequality and squalor. Fuelled as it is by new science and new technology, there is no limit to demand in the industrialized world, where the way of life of the rich is also the goal to which not only their poorer brethren but also the thousands of millions who inhabit underdeveloped countries aspire. But industrial and agricultural production have their 'external diseconomies' — for rich as for poor, for countries as for individuals. Effluents have got to be got rid of, whether by being discharged into the air, or poured into waterways, or dumped as solid waste on the land. Clearing forests for agriculture is as much a necessity in the southern underdeveloped world of today, as it once was in the developed world of the north. Environmental diseconomies are part of the total cost of a production cycle, and a part whose reduction will always be an objective in a competitive market economy, as is any other part — through lower labour costs, better technology or better management. But whatever its scale or nature, the cost cannot be avoided. "The citizen pays either as consumer, or as taxpayer, or as victim . . . the calculus of who shall pay for what improvement is *the* political issue at the core of any policy designed to deal with hidden subsidies and external diseconomies" (page 95 of ref. 2).

The political factor is indeed overwhelming. Those who achieve power in industrialized societies by way of the ballot box have no wish to make life more difficult for their supporters than it inevitably is in a competitive world. From a global point of view, how, for example, are the richer countries to choose between helping alleviate poverty

in the poorer ones or attending to the environmental dangers which unsafe nuclear reactors threaten? Our television screens constantly remind us of the poverty, starvation and pollution that affect the underdeveloped countries. Yet we take in our stride the fact that in a free market economy, circumstances may make it necessary to take hundreds of thousands of acres of land out of production in the fertile north — with farmers being compensated by the taxpayer for leaving fields to lie fallow. Governments accept that billions of dollars need to be spent to dispose of nuclear waste and to make some existing nuclear power stations safer than they are. But with the world in recession, how is the choice to be made between starving people in Ethiopia and possible nuclear fallout?

What both Stockholm and Rio have shown is that it is a waste of time to try to negotiate on national, international, global, short-term and long-term economic environmental problems as if they constituted a coherent and a similar package for all concerned. The British prime minister, John Major, stopped on his way to Rio to try to persuade Bush to change his mind about signing a draft convention on biodiversity, despite the fact that the US president had more than once made it clear that his country would not enter into an open-ended commitment that it could not honour. Had the president been more fully briefed he might have told his visitor that despite the many American forests that had been laid waste over the years, and the wildlife that had been destroyed, the United States has a record in the field of environmental protection and conservation that is second to none. Ting Weu Lian of Malaysia, the forestry expert for the developing countries, declared that "we are certainly not holding our forests in custody for those who have destroyed their own forests and now try to claim ours as part of the heritage of mankind". And the promise about the

new British initiative regarding biodiversity which was to be revealed at Rio turned out to be the 'Darwin initiative' — a move to coordinate British expertise, with 'substantial' new money, in monitoring the diversity of plant and animal species. Who briefed Major to declare this 'initiative' I cannot think. What was it that they imagined the Natural History Museum and Kew (among other institutions) had been up to all these years if not just that? And now systematists are waiting to discover how much new money the word 'substantial' means as they set about extending the work on which they have been engaged for well over 100 years.

Rio showed that substantive inter-governmental agreements on specific matters cannot emerge from a meeting of the heads or representatives of about 150 states, varying enormously in size and in economic strength, all in different stages of development, and each with its own troubles. Before the Rio conference opened, the draft treaties on global warming and biodiversity were the only two specific issues that had been considered in depth, and even they had failed to find favour with the richer countries — the United States in particular — all of which would either directly or indirectly have to foot the bill. A proposal that there should be a meeting next year for countries to report on the new measures they have embarked on to improve the environment got nowhere, with some of the most densely and heavily populated states, among them India, refusing to agree.

Had they lived to see the day, I am sure that Ward and Dubos would have been dismayed by the goings-on at the conference. But in so far as Rio, like Stockholm, has helped make the people of the world increasingly aware of the importance of environmental issues, it obviously helped. Undoubtedly there have also been some significant, even if patchy, environmental improvements in the years that separated the meetings. In the richer, more advanced parts of the world, atmospheric pollution due to the burning of fossil fuels has been reduced, and rates of population growth have declined in several countries where average incomes have increased. Although improvements have occurred in some areas they have sadly been matched by further deterioration elsewhere.

The poor, who are "both victims and agents of environmental damage", and of whom the World Bank estimates more than a billion live in abject poverty, make up most of the world's present population of 5.5 billion, which by the year 2030 is expected to reach some 9 billion, before stabilizing between 10 and 12.5 some 20 years later (UN figures). Ninety per cent of the increase will be

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taking place in the underdeveloped countries, by which time one-third of the total world's population will be living in countries with average densities of more than 400 people per km². This is today's figure for the wealthy Netherlands; it is twice as much in poverty-stricken Bangladesh. By 2025 the figure could double for Bangladesh, but there is no reason to expect any increase in the Netherlands. The amazing increase in the population of the poorer countries over the past few decades is an indirect consequence of the advances in medical and agricultural science in the advanced world.

According to a recent WHO estimate, the global fertility index has fallen in the past 20 years from an average of 6.2 children per woman to 3.9, with the rate of fall varying greatly among different peoples. It is generally believed that fertility rates in the poorer countries are high because so many children die when young, with those who reach maturity constituting a form of social insurance for the old. It is, however, striking that in sub-Saharan Africa, the fertility rate has remained unchanged over the past 25 years at about 6.5 births per woman, a rate much higher than in some parts of the world where the population has corresponding average levels of income, life expectancy and female education.

There have been other improvements since *Only One Earth*. To refer again to the 1992 World Bank report, over the past 25 years, "average consumption in developing countries has increased by 70 per cent in real terms; average life expectancy has risen from 51 to 63 years, and primary school enrolment rates have reached 89 per cent". But these are global averages, and the extent to which they have improved, is due mainly to what has been happening in India and other parts of southern Asia. In contrast, since the mid-eighties, all the bank's measures show that poverty has worsened in sub-Saharan Africa, in the Middle East, in North Africa, in Latin America and in the Caribbean. A billion people still do not have access to safe drinking water, and more than twice that number to modern methods of sanitation — this despite the fact that during the 1980s water was provided for some 700 million people in developing countries, and sanitation to an extra 400 million. These improvements failed to keep pace with the growth of population.

Were the world's population to be growing more slowly than it is, the problem of reconciling the protection of the environment with social and economic development would clearly be easier. The trouble is that no better or more effective methods of birth control than those now available are within sight. Despite widespread efforts to assist countries that have instituted

family-planning programmes, and of the successes that have been achieved, only a supreme optimist could believe that current rates of social and economic development in the world's poorest countries are likely to be paralleled by reciprocal falls in birth rates. Even in India where there has been so much improvement, widespread illiteracy is still a hindrance to the understanding, let alone acceptance, of birth-control measures. There is also a considerable unsatisfied demand for contraceptive pills and condoms in those countries where the population has already received the message, that is to say, where enough resources have been directed to

areas. Fifty years from now two-thirds will be inhabitants of mega-cities — with all the associated horrors of air pollution, inadequate water supply and sanitation. Since Stockholm, migration from the countryside into the cities has continued at an increasing pace all over the world, and as Ward and Dubos recognized, the growth of cities has everywhere resulted in urban and then suburban decay. It is as evident in Liverpool and Los Angeles as it is in Calcutta and Rio. It is to be seen everywhere.

Nothing can stop the urge to social and economic betterment that now sweeps the people of the underdeveloped countries. One need only turn one's mind for a second to what is now happening in South Africa to realize how powerful the urge is. What we also need to keep in mind is that many, if not most, of the rare species of animal and plant whose extinction the draft biodiversity treaty is designed to prevent, are native to underdeveloped countries, whose first priority, as the preamble to the biodiversity treaty acknowledges, must be social and economic development, leading to the eradication of starvation, poverty and disease. The fact that the safeguarding of biodiversity is a desirable goal for the whole world is meaningless to the tens of millions who live on the edge of starvation. Another fact we need to remember is that the environmental devastation which now attracts so much attention is not something that started yesterday. It began in the north in the seventeenth century with the first industrial revolution, and went on increasing steadily as factories spread and then became exacerbated by the new agricultural practices, particularly the use of agrochemicals after the First World War. Environmental degradation in the north has been going on for 300 years. Rio was a warning that the underdeveloped countries are not ready to spend centuries in their efforts to reach standards of living equivalent to those that now prevail in the north.

Exactly what sustainable development means in the world as we know it, as opposed to that of the planning theorist, I am not sure. But what has become clear is that social and economic growth in the developing countries, or indeed in the world as a whole, is not likely to be limited because of the possibility that non-renewable resources will run out. According to the World Bank (see also refs 4, 5), the evidence "gives no support to the hypothesis that marketed non-renewable resources such as metals, minerals and energy are becoming scarcer in an economic sense". Figures for reserves today are higher than they were 20 years ago "because potential or actual shortages are reflected in rising market prices, which in turn have induced new

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Burning the Amazon forest — 10 per cent has been cleared in the past 10 years.

the improvement of education. The estimate given in the World Bank's most recent report is that family-planning aid now consumes \$5 billion a year, and that it needs to rise to \$8 billion if the increase in world population is to be kept within estimated limits.

The dilemma that faces rich and poor alike is that we have managed to free ourselves from the constraints that limit the growth of plant and animal life — namely, the climatic and other limitations of the habitat within which species have evolved and to which they became adapted; disease; and interspecific (and to a limited extent intraspecific) predation. While not directly dependent on the food chain which the whole living world constitutes, we are nonetheless the most dominant predators of all. There are some who argue that, for man, war constitutes a form of intraspecific predation. In relative terms, however, it has not as yet been as big a killer as have some diseases, and as AIDS might be, or as all-out nuclear war certainly would.

Most of mankind now lives in rural

Mark Edwards/Still Pictures

discoveries, improvements in efficiency, possibilities for substitution, and technological innovations". Nor is there any overall shortage of food in the world. Food production has doubled over the past 25 years — a rate of increase greater than that of the world's population. Nine-tenths of the doubling was due to scientific and technological developments in agricultural and food science, and only one-tenth to cultivating more land. Deforestation, in order to increase the acreage that is devoted to agriculture, is positively correlated with the growth of population, and quite apart from the consequent loss of the habitats of wild creatures and plants, the process is often associated with overgrazing and the depletion of water supplies. This sequence of events is certainly not novel. It was happening in northern Africa and Mesopotamia in the time of the pharaohs. What we have to hope is that since we know how badly the process has so often ended in the past, the process of extending agricultural production in underdeveloped countries will be better managed in the future.

The representatives of the poorer countries who attended the Rio assembly were obviously aware of the mistakes that have marred so many World Bank and other big development projects in the past. But as they also made clear, they want to help determine for themselves what course future developments should take, as opposed to being told. They justly claim sovereign rights to their own natural resources and can be expected to demand an adequate share of whatever profit industrialized countries expect from investments they make in them, or alternatively to be compensated if for environmental reasons they withhold from their exploitation.

Doubtless every developing country has its own political and environmental problems. Some they share with others. For example, hundred of millions of the world's poor suffer more from indoor pollution caused by the burning of wood, straw, or dung for cooking and heating than they do from any outdoor atmospheric pollution. All of them also know that their poverty cannot be cured except with the help of the rich, developed countries. They hear of vast amounts of money that are going to be made available to help them — for example, contributions by all EC countries of 0.7 per cent of GNP to the UN aid programme, and contributions to the 'global environmental facility' that has recently been set up within the World Bank — with estimates of \$125 billion a year needed to carry out its *Agenda 21*, the programme accepted 'in principle' at Rio. But experience has also taught the poorer countries that promises are more frequently hedged with qualifications than

matched with immediate gifts of money. They also know that the path of development is signposted with problems of debt and corruption. It is estimated that debt repayments by developing countries to their northern lenders over at least the past decade have exceeded new loans by about \$30 billion a year.

Scientists as such play no part in deciding on the political and economic issues that determine what levels of aid should be provided in the development of the poorer countries, or what private investments they should attract. Here their responsibilities lie in the parts they play as, for example, hydrologists, agricultural experts or mining engineers. The scientist's special and global responsibility in matters environmental is to increase our understanding of the dynamics of climatic change. The likelihood of significant changes in global temperature or the depletion of the ozone layer are matters that affect all people equally. Some cynics say that the north's essential concern with the environment of the south is in the latter's contribution to the emission of greenhouse gases. Even if that were so, what would matter is the urgency which the north accords the help it must give southern countries in their efforts to develop educationally, economically and socially, in ways that help restrain the growth of population and do least harm to their own and to the global environment. What matters equally is the speed with which the north succeeds in repairing the environmental damage it has caused in the past, the speed with which it now develops sources of power that do not damage the environment (including safer nuclear power) in ways of conserving energy and other resources, and in all other ways that help reduce the continuing pollution of the atmosphere, waterways and seas.

What also matters is that those scientists who are directly involved in the study of the factors that are concerned in climatic change do their work in strict accordance with scientific method, so that agreed and unambiguous views about the environment can be provided to the governments of their respective countries and to the relevant international institutions. For example, the extent to which global temperatures might be increasing is still not an agreed matter. Some have spoken about rises of as much as 3 to 4 °C, some of less than 1 °C. There are differences of view about timescales. From the point of view of governmental action it matters greatly which estimates should be accepted, what rises, if any, in sea-level to prepare for, and whether agricultural production is likely to be significantly changed geographically, difficult as it undoubtedly would be for scientists to achieve general agreement. A slight error could lead

either to a delay in taking action or to precipitate action, with unpredictable and even disastrous consequences.

Today there are some who argue that the transfer of capital to the developing countries will result in more environmental harm than good, that it will increase industrial demand and pollution, and that in any event it leads to economic colonialization and to the elimination of self-reliant national cultures. These arguments may be sincere, but it is nonetheless true that nothing can stop the drive for social advancement that now pervades the developing countries.

Ward and Dubos had hoped that a cessation of the superpower nuclear arms-race would release resources which could be diverted to environmental protection. There, too, they would have been disappointed. That particular race has ended, but with the break-up of the Communist states, the world is in greater political turmoil than it was 20 years ago, with the arms trade flourishing as never before in a free and competitive world market. Whether elimination of world poverty and protection of the global environment are in fact compatible with the workings of such a market, only our successors will know.

In one of a series of public lectures given in Stockholm as part of the 1972 conference, I argued against taking too gloomy a view of our chances of managing our environmental problems. Ward, in her address⁶, urged the need to collect accurate knowledge of the environmental changes taking place in global climate, and in the environmental conditions of different countries. She also said that we should "not linger too long on the newly recognized fact that our total natural system — in the biosphere of air and soil and water — could be irretrievably upset by man's activities. Of course, we have always known that we could chip away at it — here a forest destroyed, there a desert spreading. But the idea that the two great reservoirs of life, our airs and oceans, could enter on a general trend toward mortal damage — that is completely new."

It is no longer new. It is a fact that is understood by all literate people. Because it is, I continue to believe that in the end, man will not destroy the only planet which, to our knowledge, is the habitat of what we call the living world.

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Consciousness on the scientific agenda

The convention that the concept of consciousness is so ill-defined that it lies beyond the reach of serious study seems to be losing ground. But definition is still some way off.

How does the brain generate conscious experience? Twenty years ago, most scientists thought this a question for the philosophers; and most philosophers regarded it as a symptom of some kind (though what kind never became clear) of deep linguistic confusion. In sharp contrast to this picture, there was a large measure of agreement among both scientists and philosophers at a recent symposium* that not only is there a real problem of consciousness but that it is a scientific problem and that the time has come for scientists to tackle it.

Hardly anybody today doubts that consciousness is in some way a product of the brain, a product that is intimately connected with the brain's role in behaviour and the processing of information. Cartesian dualism — the notion that brain-stuff and mind-stuff are essentially separate, though able to communicate with each other — has virtually no contemporary followers. Dualism has not, however, vanished without trace. It has left behind the notion of a 'Cartesian theatre' (located by Descartes himself in the pineal gland, the place where brain-stuff and mind-stuff were supposed to communicate): a privileged site at which brain events enter into conscious awareness, those happening elsewhere remaining in a kind of outer darkness.

This notion came under strong attack at this month's meeting. Instead, it was proposed (M. Kinsbourne, Sargent College, and D. C. Dennett, Tufts University, Massachusetts) that consciousness is a property of whatever pattern of distributed neural firing is (in some still obscure sense) 'dominant' at any particular time. This proposal, however, raises the so-called 'binding' problem: what feature of neuronal activity unifies the ramified patterns of individual neuronal events that take place in millions of separate cell-bodies and in even more millions of synaptic junctions at any given instant? And there is a temporal as well as a spatial aspect to the binding problem: neuronal events take place on a timescale measured in milliseconds, whereas the contents of consciousness have an apparent duration at least two orders of magnitude greater. (Perhaps the only important gap in the coverage of topics at the symposium was a consideration of recent proposals^{1,2} that a solution to the binding problem may unexpectedly be provided by quantum mechanics.)

Also more or less absent from the contemporary scene are behaviourism and its

philosophical equivalent, positivism: the notion that, because only behaviour, not conscious experience, is amenable to direct observation, talk about conscious events has no place in the world of science. There has also been a more radical behaviourist view that holds, on the same grounds, that consciousness does not really exist. (One is tempted to add: it is 'just a figment of the imagination'; but that leads to some rather obvious problems.)

I once asked such a radical behaviourist what, on this view, is the difference between two awake individuals, one of them stone deaf, who are both sitting immobile in a room in which a record-player is playing a Mozart string quartet? His answer: their subsequent verbal behaviour. Mercifully, there were no radical behaviourists at the symposium.

But behaviourism too has not vanished without trace. Its modern offspring is a vigorous form of functionalism, which enshrines as the touchstone of the presence of consciousness the Turing test: if you feed symbols (for example, a string of English words) to a machine (or, more particularly, to a digital computer) and a human being and get symbols back from each of them in reply, and if you cannot distinguish between the machine and the person on the basis of their replies, then, if the human being is conscious, so is the machine. On this view, when the machine correctly uses the symbols for 'red', 'pain', 'itch' and so on, it makes no sense to ask further whether the machine has the sensory experiences ('qualia') that belong to these terms.

When this view is held strongly, it is known in the trade as 'strong AI' (artificial intelligence). A famous attack on strong AI — the 'Chinese room argument' — was made some years ago by John Searle³ (University of California, Berkeley). Essentially, the argument demonstrated that computers have only syntax, not semantics; that consciousness is permeated with semantics (one is typically conscious of things such as 'a red rose situated on a table over there'; indeterminate itches and the like are the rarity); so computers cannot, just by computing transformations of symbol strings, achieve consciousness.

On the whole, Searle seems to have won his argument: at any rate, nobody at the meeting made a serious attempt to refute it. Instead, the current form of functionalism goes beyond the mere digital computer, allowing it now to have limbs and sense organs (that is, the computer becomes a robot).

Moreover, proponents of the contemporary functionalist approach, unlike the earlier radical behaviourists, are willing — indeed, eager — to take account of what is known about events in the real brain that lie between input (stimulus) and output (response).

Dennett, for example, argued that one can already take what is known about the way the brain codes and recodes visual stimuli, put it together with existing data from psychophysics and use the knowledge that results to predict, successfully, new visual phenomena, such as illusory experiences. One can even 'inject' such experiences into the brains of experimental animals, as demonstrated in elegant experiments (W. T. Newsome, Stanford University) in which monkeys responded (behaviourally) to microstimulation of a circuit encoding a particular direction of motion in the same way that they had been trained to respond to an external stimulus having the same directional value processed by the retina and what lies behind it.

What more than this, Dennett asked, do we need? There is no theoretical problem about consciousness, we just have to go on gathering new data, and when we have all the details of what goes on in the brain and how this brain activity interfaces with the environment, that will be in and of itself a complete scientific account of consciousness.

Some, however — probably the majority at the meeting — remain unconvinced that it is going to be so simple. For them, more than just dedicated gathering of experimental data will be needed. What is needed is, rather, a new theory (one comparable with, say, the theory of heat for the relation between the gas flame and the boiling of the kettle) that will render the relations between brain events and conscious experiences 'transparent', to use T. Nagel's (New York University) felicitous term. A series of brute correlations would not suffice. This, after all, is the standard set in all other domains of science, so why not here? This new theory is at present unimaginable, but only in the sense that no-one could have imagined relativity or quantum mechanics before *they* were invented, not because we are dealing with the unknowable or a bad language habit.

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Folding in the egg white

Roger H. Pain

PROTEINS seem to find it easy to fold into their three-dimensional conformation — the difficulty is the scientist's, in failing so far to understand how they do it, let alone predict either the pathway or the end state. Three of the more important questions facing the protein folder are these. First, at what stage on the folding pathway does the protein become compact? Second, how cooperative is the folding process? And third, to what extent is there a single pathway? On page 302 of this issue¹ Radford *et al.* implicitly address these matters, and shed some more light on the folding pathway of a familiar protein, hen egg-white lysozyme, using two of the newest techniques (pulsed hydrogen-exchange labelling and stopped-flow circular dichroism).

There are few probes of the different features of protein conformational change in solution that are effective on the millisecond scale, but as many of them as possible need to be applied to a given protein if unambiguous conclusions are to be reached. The use of two-dimensional NMR to identify which amide protons are protected against exchange with D₂O as folding proceeds² provides a detailed insight unobtainable by other techniques. The temptation to relate protection directly to the formation of secondary structure has, however, to be resisted.

A good example of this is seen in the paper by Radford and colleagues, where circular dichroism (CD) shows protection to be preceded by the formation of optically active, secondary structure. Protection is a quantitative rather than a qualitative measure, depending on the stability of the secondary structure and the rate of the chemical exchange process during the labelling pulse. Thus a poorly stabilized helix formed early during folding may well not exhibit measurable protection. Kinetics of increasing protection observed in such a case will therefore be indicating the accumulation of tertiary interactions that lead to stabilization of the helix.

Stopped-flow CD in the far ultraviolet, by contrast, gives an indication of secondary structure formation but not of quantitative stability. In addition to the peptide bond, other chromophores such as aromatic and disulphide groups can give rise to ellipticity in this region of the spectrum and it is difficult to resolve the different potential contributions. In following the acquisition of secondary structure in lysozyme, Radford *et al.* found an overshoot of ellipticity at 225 nm. The problem is to distinguish be-

tween transitory formation of more secondary structure than exists in the native state on the one hand, or temporary asymmetry of a disulphide bond or of the environment of an aromatic residue on the other. It would be interesting to know whether the complete spectrum of the higher ellipticity intermediate corresponds to a defined mix of secondary structure components or not.

Near-ultraviolet CD arises from the asymmetry of environment of aromatic residues, the band at 289 nm corresponding to tryptophan. This provides a

by tertiary interactions. This points to a degree of compaction of the α -domain in the early folding intermediate, probably, as in the native structure, as a result of hydrophobic stabilization. Detailed support for this kind of mechanism is provided by the demonstration that two tryptophans are involved in helix-helix interactions in the α -domain. Further, the fact that most of the amides in the α -domain helices are strongly protected, coupled with the absence of near-ultraviolet ellipticity, suggests to the authors that this domain is in the form of a 'molten globule' at this stage.

The kinetic phases corresponding to increasing development and stabilization of secondary structure must also reflect increasing tertiary interactions, packing

Kinetic phases observed during folding of lysozyme¹

Time constant	Probe	Structure monitored
Submillisecond	Far-UV CD: θ_{225}	Backbone secondary structure (~ 65%)
5 ms	Amide protection	α -domain secondary and tertiary structure
8–10 ms	Amide protection and near-UV CD: θ_{289}	β -domain secondary and tertiary structure
30–40 ms	θ_{225}	Uncertain
60 ms	Amide protection	α -domain helix stabilization/tertiary structure
100–800 ms	Amide protection/ θ_{225} (decrease)	β -sheet in β -domain secondary/tertiary structure
285 ms	θ_{289}	Tertiary structure

very sensitive index of unperturbed native tertiary structure, and, since different residues can make rather different contributions, can constitute a rather precise index of localized conformational change if assignments have been made³. Unfortunately this is not usually the case. Despite the fact that neither the amide protection nor the circular dichroism techniques individually allow a complete picture to be obtained, used together they can provide complementary information; it is this strategy that has been adopted by Radford *et al.*

Like most other proteins, whether they possess intact disulphide bonds or not, a number of kinetic phases are observed when lysozyme is allowed to fold, as summarized in the table. The use of the two complementary techniques enables these phases to be assigned to the development of structure in different parts of the molecule. The native structure of lysozyme is folded into two domains, one (α) containing five helices and the other (β) the two β -sheets, a 3¹⁰ helix and the large loop region. The two domains have previously been shown to fold independently⁴.

How do these results bear on the first question, that to do with the stage at which the protein becomes compact? Formation of substantial proportions of the relatively short helices in a protein such as lysozyme requires stabilization

and compactness. Binding of the hydrophobic probe anilino-naphthalene sulphinate has also been taken to support collapse of the polypeptide chain⁵. Although the kinetic molten globule has been shown to be almost as compact as the native state⁶, the point on the pathway where it achieves this degree of collapse awaits a more direct and quantitative measure of compactness. This support, albeit indirect, for collapse at an early stage in the folding pathway will however encourage those who hold that a hydrophobic collapse is required for the formation of substantial amounts of secondary structure.

Turning to the question of cooperativity, the results of Radford and colleagues show clearly that, in certain aspects, lysozyme folds in a manner that is far from kinetically two-state. The early stabilization, and possibly the formation, of secondary structure in the α -domain is significantly faster than in the β -domain. It is interesting however that in the later

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phases the similarity of rates of acquisition of near-ultraviolet ellipticity — with likely contributions from the α -domain — and of increasing amide protection in the β -domain suggest an increasing cooperativity as the protein approaches the native state.

It is particularly notable that the biphasic kinetics of amide protection reported in these experiments can only be accounted for by there being parallel pathways on which amide protection is achieved at different rates. Increasing the rate of amide labelling by raising the pH at the pulse step had no effect on the degree of protection, as would be expected if there were a simple sequential mechanism. The rates are apparently not

consistent with a proline isomerization mechanism, but could be the result of other kinds of delay such as association of partially folded intermediates. In such cases the pathway following the delay step would be identical for each phase as in proline isomerization. It is intriguing to speculate that if collapse were to occur in globules separated by appreciable energy barriers, the succeeding pathways could in principle be different — reminiscent of the jigsaw model of protein folding⁷.

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CHEMICAL KINETICS

Probing the transition state

Ian W. M. Smith

CHEMISTS of all kinds retain a fascination for transition states, those elusive and transitory intermediate structures through which molecular systems must pass, as bonds break or rearrange and a chemical reaction occurs. Transition states possess some of the properties of stable molecules. In particular, there are quantum energy levels associated with all the motions which are orthogonal to the crucial motion which carries the system through its transition state and forward to products. Until very recently, however, it had been impossible to observe the pattern of these energy levels and hence to deduce, from experiment, the molecular structure at a transition state. The evolving nature of species

undergoing chemical or photochemical transformation can be observed by performing kinetic spectroscopy on the femtosecond timescale¹. However, even this method does not provide information about the unique transition state for a reaction.

An alternative approach which probes the transition state directly has just been reported by Bradley Moore and colleagues² from the University of California at Berkeley. Their method rests upon the prediction of the 'RRKM' theory of unimolecular reactions that the rate constants $k(E)$ for microcanonical assemblies of molecules (molecules prepared with the same internal energy E) do not vary smoothly with E but in a stepwise fashion, as quantum levels in the transition state become energetically accessible and provide additional 'doorways' through which reaction can occur.

In quantitative terms, RRKM theory (named after its devisors) predicts that the energy-dependent rate constants depend on two factors: $\rho(E)$, the density of states (or number per unit interval of energy) in the reagent molecule, and $W^\ddagger(E)$, the number of vibrational levels of the transition state with energy less than E , according to the equation

$$k(E) = W^\ddagger(E)/h\rho(E)$$

where h is Planck's constant. Generally the density of states $\rho(E)$ varies quite slowly, so that as E is increased above its minimum or threshold value E^0 for reaction, $k(E)$ should increase by approximately $1/h\rho(E^0)$ each time one reaches an energy at which another quantized level in the transition state is accessible.

The success of Moore's experiments which provide the first experimental

verification of the discontinuous form of $k(E)$, rests both on a well thought-out choice of molecular system to study and on certain critical features of his experiment. They ensure that the steps in $k(E)$ are both deep enough and wide enough to be resolved and also that the quantum level structure is not washed out by thermal averaging over a variety of similar but not identical $k(E)$ curves.

The experiments measure rate constants for the dissociation of vibrationally excited CH_2CO close to the threshold for production of CH_2 in its $^3\text{B}_1$ state plus CO. The excited molecules are prepared by irradiating ketene molecules in a supersonic jet expansion with monochromatic radiation from a tunable dye laser. The excited molecules, or some fraction of them, undergo rapid intersystem crossing to a triplet state from which dissociation takes place (Fig. 1). The formation of products is observed by using a tunable vacuum-ultraviolet laser to detect CO molecules by laser-induced fluorescence (LIF), and rate constants in the range $1\text{--}20 \times 10^6 \text{ s}^{-1}$ are measured by scanning the time delay between the photolysis and probe lasers.

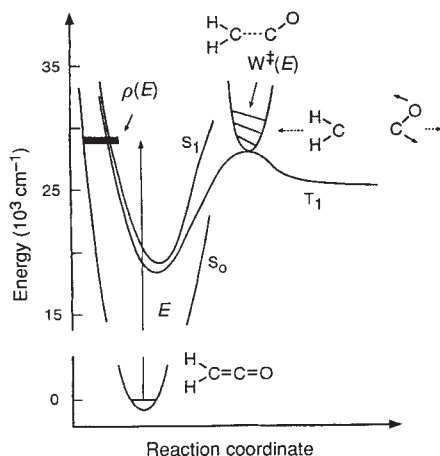


FIG. 1 Energy curves representing the dissociation of triplet ketene, with the 'reaction coordinate' representing the C-C distance. Curves represent the potential energy for the singlet (S_0 , S_1) and first excited triplet (T_1) states. Quantum levels in the transition state are represented schematically above the maximum on the T_1 curve. (From ref. 2.)

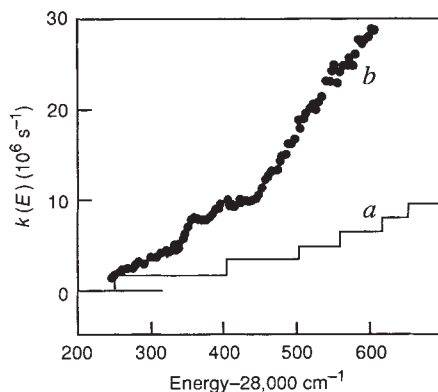


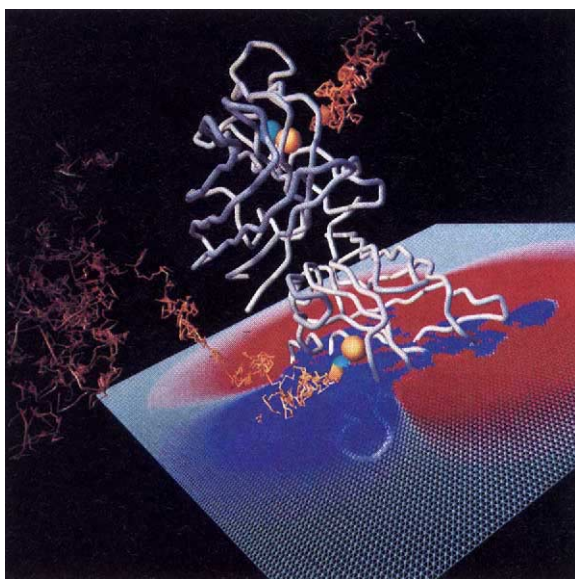
FIG. 2 a, An idealized form of the $k(E)$ curve for the dissociation of non-rotating triplet ketene. The quantum levels in the transition state are calculated (neglecting anharmonicity) from the frequencies calculated by quantum chemical methods⁶. b, The measured rate constants $k(E)$ of Lovejoy *et al.*² for ketene molecules excited by specified photon energies.

In what must be described as an experimental *tour de force*, this type of experiment has been repeated many times with slightly different excitation wavelengths to construct $k(E)$ versus E curves (Fig. 2).

In addition to the measurements just described, somewhat simpler experiments were performed to record what are termed PHOFEX (photofragment excitation) spectra. The frequency of the probe laser and the time delay between the light pulse from this laser and the pulse from the laser photolysing the ketene are both fixed, and the LIF signals from CO are registered, as the

Quicker reaction by design

THE speed with which superoxide dismutase (SOD) catalyses the conversion of superoxide anions ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) is so fast that the reaction is limited only by diffusion of the $O_2^{\cdot-}$ anion into the active site of the enzyme. Getzoff *et al.* (page 347 of this issue) have now improved this apparently 'perfect' enzyme by increasing the net positive charge in the vicinity of the active site, hence facilitating diffusion of $O_2^{\cdot-}$ to the active site. The computer model shows the paths of incoming $O_2^{\cdot-}$ (thin gold lines) under the influence of brownian motion and the electrostatic field of human (Glu 133 $\rightarrow$ Gln) SOD. The grid displays the electrostatic potentials surrounding the positively charged (blue) active site and the negatively charged (red) protein at points on a plane passing through the dimeric SOD molecule (α backbone, white tubes). The paths of $O_2^{\cdot-}$ (brighter as they approach the enzyme) terminate when a reaction occurs (gold octagon) at one of the two active sites (Cu and Zn ions, the gold and blue spheres respectively). For computational methods see the legend to Fig. 3 of the paper. The computer graphics model was made by M. Pique, J. Talner and E. Getzoff with the Application Visualization System software.



frequency of the photolysis laser, and hence the photon energy supplied to ketene molecules, is varied. The PHOFEX signals showed steps matching those in the $k(E)$ curves, consistent with the expectation that it is changes in $k(E)$ which largely determine the structure in the PHOFEX spectra.

What are the crucial features alluded to earlier that allow Moore and colleagues' experiment to uncover the structure in the transition state associated with ketene dissociation? The first, which is crucial in many modern experiments in spectroscopy and reaction dynamics, is the cooling of the reagents provided by the jet expansion. Strictly, the microcanonical rate constant depends not only on the internal energy of the reagent but also on its state of total angular momentum, J , as this must be retained during unimolecular fragmentation. Jet cooling severely restricts the J states populated by ketene molecules, with the result that the averaging over different $k(E, J)$ curves is minimal and it does not obscure the structure in $k(E)$ resulting from the pattern of quantum levels in the transition state.

The second, and perhaps even more important aspect of the study is related to the special features of the ketene dissociation. As Fig. 1 indicates, there is a well-defined energy barrier along the path leading to the dissociation products.

This is not always the case for a unimolecular reaction, and it leads to a transition state which is fairly tightly constrained and which consequently has rather well-separated quantum levels. The lowest frequency modes of motion, which lead to the closest-spaced quantum levels and hence to the most closely spaced steps in $k(E)$, are the bending of the C–C–O angle and the torsional motions of the CH_2 and CO moieties around the breaking C–C bond. The torsional motion becomes an internal rotation at energies of about 400 cm^{-1} . As a result, energy levels associated with this motion become much more closely packed and this may be the reason for the rapid, and essentially unstructured, increase in $k(E)$ at the higher values of E which were examined.

Another interesting characteristic of the results is that, in principle, the rate constant for dissociation at threshold should correspond to that associated with passage through the single, lowest level in the transition state, so that $k(E^0) = 1/h\rho(E^0)$. Consequently, it should be possible to compare the density of reagent states at threshold derived from the experimental value of $k(E^0)$ and the value estimated from a knowledge of the frequencies and anharmonicities of the vibrations of CH_2CO . In practice, there is some uncertainty about the degeneracy of the transition-state quantum

levels. But Moore and coworkers performed experiments on CD_2CO , as well as on CH_2CO , and find a fourfold reduction in $k(E^0)$, to be expected because of the greater density of states in this isotopically substituted molecule.

The results of the experiments demonstrate clearly the quantization in the transition state of dissociating ketene. A logical follow up will be to compare the experimental results with detailed calculations of $k(E)$, based on accurate computations of reagent state densities and transition state quantum levels. In this way, and perhaps with still higher resolution in the experiments, it may be possible to uncover non-RRKM behaviour³ associated, for example, with some breakdown in the entirely statistical base of RRKM theory.

There is little doubt that Moore's work will stimulate others to similar efforts. Indeed, already a paper⁴ from Wittig's laboratory at the University of Southern California reports observations of the steplike variation of $k(E)$ in the photoinitiated decomposition of NO_2 . The small size of this reagent, and consequent reduction in $\rho(E)$, means that its unimolecular lifetime just above E^0 is in the picosecond range, requiring the use of subpicosecond laser pulses for excitation and observation (in this case of NO). The size also brings an important benefit, as it simplifies the quantum level structure in the transition state, although the interpretation is clouded by extensive mixing of electronic states at energies sufficient for dissociation.

Other reactions which suggest themselves as targets for this kind of study are those with tightly constrained transition states, so that the quantum levels in the transition state, at least close to threshold, are widely spaced. Four-centre unimolecular decompositions, such as the elimination of hydrogen halides from halogenated alkanes and alkenes, are prototypical of such reactions. They occur via transition states which should have easily resolvable structure⁵. Whether the other conditions, which have been so successfully satisfied in the experiments in Moore's and Wittig's laboratories, can be satisfied in these other molecular systems remains to be seen. □

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A case of missing water

John T. Andrews

As the globe warmed and massive ice sheets melted at the end of the last ice age, sea level world-wide rose by around 120 metres¹. The first 100 metres or so of the rise were accounted for by melting of ice sheets in the Northern Hemisphere² (18,000–7,000 years ago), leaving the last 25 metres or so to be supplied by the Antarctic ice sheets less than 9,000 years ago, or so it is assumed. But new measurements by Colhoun *et al.*, on page 316 of this issue³, show that the Antarctic contributed at most 2.5 metres to sea level, and maybe as little as 0.5 metres.

Keeping track of the ice sheets and sea level could be a relatively straightforward matter were it not for the flexibility of the Earth's crust. But allowance has to be made for the flexure of the continental crust under the weight of the ice sheets and for depression of the sea floor as the mass of sea water increases, which leave a constantly shifting base line. For the Northern Hemisphere, the change in relative sea level has been tracked at many coastal sites, enabling, for example, Tushingham and Peltier to construct highly detailed histories² for the regression of the ice sheets and advance of the oceans for the end of the last ice age.

But in the Southern Hemisphere, matters are not so good. For Antarctica itself, only one relative sea level curve has been determined, and to constrain their model Tushingham and Peltier had to rely on data from New Zealand and other remote Pacific sites. So in spite of Antarctica's patent importance in these matters (its current ice sheets hold enough water to raise sea levels a further 60 metres), modellers have very little evidence to go on.

Colhoun *et al.* use the ages and elevations of raised beaches in the Ross Embayment and on Eastern Antarctica to develop their version of the deglaciation there. These are former beaches that, because of unloading of the ice sheets, have been raised up to tens of metres by the flexing crust. Although no single beach yields a relative sea level curve, collectively they offer an insight into the end of the last ice age.

The authors show that the retreat of the Antarctic ice sheets was already well underway 11,600 years ago, well before the 9,000 years previously imagined, and was completed by 6,000 years ago. (With a detailed calibration now available⁴, it is possible to convert the radiocarbon dates of Colhoun *et al.* confidently into calendar years.) By comparison with the highest raised beaches (marine limits) in the Northern Hemisphere, those in

Antarctica are relatively low — 30 metres, at most, above sea level. In Greenland, for example, elevations in excess of 80 metres are quite common⁵. It is with this contrast in mind that Colhoun *et al.* argue that the Antarctic ice sheets were thinner at the last glacial maximum than others believe. Otherwise the marine limits, with greater rebound of the crust, would have been raised higher.

The estimates are clearly controversial. They contradict, for example, interpretations of marine seismic and core data. But over the next three years, the West Antarctic Ice Sheet Project⁶ (WAISP) may be able to verify them. The surprising conclusions, however, underline the difficulties in elaborating the details of glacial–interglacial history. Indeed, only recently Lambeck and Nakada tried to rewrite the story of the interglacial period immediately before the last ice age⁷. Although many have accepted that the sea level was 5 metres higher then than now, suggesting that either Greenland was entirely deglaciated or that the West Antarctic Ice Sheet had disintegrated, Lambeck and Nakada argue from analyses of raised coral reefs that the ocean volume was no different from today. Moreover, the warm period lasted from 135,000 to 120,000 years ago, spanning a considerably longer period than previously believed.

The bottom line of Colhoun and colleagues' paper, that Antarctica contributed only 0.5–2.5 metres to the post glacial sea-level rise, will surely be challenged. But if the authors are right, where did the 25 metres everyone seems to expect come from? The northeastern sector of the Laurentide Ice Sheet on North America, the Franklin/Innuian Ice Sheet complex of the Canadian High Arctic or possible ice sheets across the Eurasian continental shelves might be implicated. But it is hard to believe they are the whole answer. □

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Nonproliferation theory

PHYSICISTS searching for the sixth quark, called top, may be encouraged by new calculations indicating that this is the last one that remains to be identified (Y. Iwasaki *et al.* *Phys. Rev. Lett.* **69**, 21–24; 1992). So far five types (or 'flavours') of quark are known — up, down, strange, charm and bottom. A sixth is confidently expected, if only because the first four make two matched pairs and a third pair would mirror the three members of the electron family. But are there any more? The new calculations look at what would happen to the interactions between the quarks using lattice quantum chromodynamics (dividing space into discrete points makes the number crunching tractable). With more than six flavours, it seems, the strong force would lose its most striking feature, confinement, and single quarks could be found in isolation. That would contradict all the evidence.

Skeletal skeleton

Aficionados of the cytoskeleton have always been preoccupied with defining the types of nexus between filamentous and other structural proteins — the shrouds and halyards of the cell, as they have been called — that prevail in different tissues. G. A. Porter *et al.* (*J. Cell Biol.* **117**, 997–1005; 1992) now report that in skeletal muscle the distributions of three sarcolemma-associated proteins are superimposed: dystrophin, muscle spectrin and vinculin occur in hoops around the I-bands and the M-lines of the contractile lattice and also in relatively sparse longitudinal strands. In dystrophic muscle the spectrin distribution is known to be unaffected, so the network does not depend on dystrophin. The suggestion is that the three proteins together form the system that couples the sarcolemma to the contractile machinery.

Gliding light

THE discovery of fossil teeth of a species of flying lemur from Eocene rocks in Thailand casts doubt on the affinities of other fossils assigned to this enigmatic group, according to S. Ducrocq and colleagues (*Palaeontology* **35**, 373–380; 1992). The term 'flying lemur' is a misnomer: the animals neither fly (they glide) nor are they lemurs. The two extant species belong to a single genus, *Cynocephalus*, the sole representative of the Order Dermoptera; although reckoned to be allied to primates, their fossil record is sparse and ambiguous. The new fossil, *Dermotherium major* differs from all previous contenders in that its teeth are recognizably like those of *Cynocephalus*. Now that the fossil history of *bona fide* dermopterans goes back to the Eocene, the status of the several fossil groups assigned to the Order must be in question.

Exchange rate mechanisms

Julian Downward

MUCH of the research into growth-signalling mechanisms in eukaryotic cells centres on the proteins encoded by the *ras* oncogene family. The report on page 351 of this issue by Shou *et al.*¹ will add considerable impetus to that endeavour — the authors describe the cloning and characterization of a mammalian guanine-nucleotide-exchange(-releasing) factor specific for Ras proteins, thereby delivering an example of a molecule that has been pursued by many groups for many years.

Ras proteins are members of a large superfamily of low-molecular-weight proteins which bind guanine nucleotides. They appear to couple growth-signalling molecules at the plasma membrane to intracellular targets such as the cytosolic kinases Raf and Erk which coordinate transmission of signals from the cytoplasm to the cell nucleus, leading to regulation of DNA transcription. Ras proteins exist in two conformational states

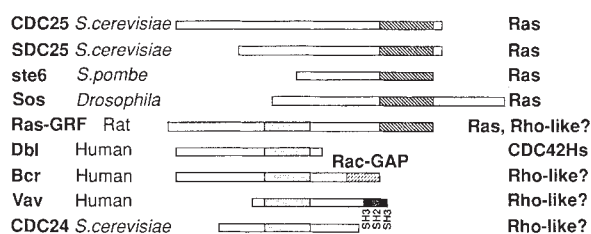
— active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP).

Within the cell, Ras is acted upon by a number of proteins that influence its activity. These proteins fall into two groups, those that stimulate the weak endogenous rate of GTP hydrolysis (GTPase-activating proteins, or GAPs) and those that stimulate the rate of guanine-nucleotide exchange on Ras (reviewed in ref. 2). Interaction with a GAP leads to Ras entering an inactive GDP-bound state, whereas interaction with an exchange factor causes release of bound nucleotide (usually GDP owing to the action of GAPs), and uptake of fresh guanine nucleotide from the cytosol (mostly GTP). Ras interaction with exchange factor would thus be expected to lead to its activation: regulation of the activation state of Ras can occur either by stimulation of exchange factors or by inhibition of GAPs. Two mammalian GAPs have been characterized in detail, p120^{GAP} and neurofibromin, the second being the product of the type 1 neurofibromatosis gene. Mammalian exchange factors, however, have been much more elusive.

In 1990, three groups reported the identification and partial purification of Ras-exchange activities from mammalian tissue extracts^{3–5}, but attempts to obtain structural information from these proteins proved fruitless. So Shou *et al.*¹ chose a different approach. They took advantage of the fact that exchange fac-

tors for Ras had been genetically identified in yeast some time ago: CDC25 and SDC25 in *Saccharomyces cerevisiae*, and *ste6* in *Schizosaccharomyces pombe*. Despite the huge evolutionary distance between these two yeasts, the sequences of their exchange factors have some remarkably conserved regions.

Shou *et al.* designed oligonucleotide primers based on the most conserved regions of these exchange factors, used them in a polymerase chain reaction on rat brain complementary DNA, and



Exchange factors for the Ras superfamily of proteins. Target GTP binding proteins are listed on the right. Regions of CDC25-like homology and of Dbl-like homology are shaded.

obtained clones of a rat brain CDC25 homologue: the full-length sequence predicted a protein of relative molecular mass 140,000 (140K) with a carboxy-terminal region similar to that of CDC25 (nearly 30 per cent over 300 amino acids). The protein, named Ras-GRF (for guanine-nucleotide-releasing factor) is also similar in sequence over this region to Son of sevenless (Sos), the homologue of CDC25 found in *Drosophila*. When this carboxy-terminal region of Ras-GRF was expressed in bacteria, it was found to possess specific and potent exchange activity towards RasH and RasN proteins but not towards closely related proteins such as Ral. Antibodies raised against Ras-GRF detect a 140K protein in rat brain membranes and cytosol. Interestingly, Gross *et al.*⁶ have shown that antibodies against yeast CDC25 cross-react with a 140K protein in mammalian brain extract.

In addition to the expected sequence similarity with CDC25, a second similarity was observed: towards the amino terminus of Ras-GRF lies a region akin to the product of the *dbl* oncogene. This part of *dbl* was recently shown to encode guanine-nucleotide-exchange activity for the Ras-related protein CDC42Hs (ref. 7), itself closely related to the Rac and Rho proteins which have been implicated in control of cytoskeletal organization⁸. The Dbl motif has also been found in the Bcr, CDC24 and Vav proteins although its activity in these cases is uncertain⁹. It is not known which

Rho-like protein, if any, this Dbl-related domain can act upon, but it is clearly possible that Ras-GRF could have two exchange-factor domains which are active for different members of the Ras superfamily.

This potential multifunctionality is reminiscent of the Bcr (breakpoint cluster region) protein, which has both a Dbl-like domain and Rac-GAP activity¹⁰, and of the p120^{GAP}/p190 complex which possesses both Ras-GAP activity and Rho-GAP related sequences¹¹. Conceivably, these molecules represent points of cross-talk between signalling pathways involving different members of the Ras superfamily, particularly, in the cases of Ras-GRF and p120^{GAP}/p190, between Ras and Rho family members.

That could explain the potent effects of growth factors, frequently activators of Ras, on cytoskeletal structure, which is regulated in part by Rho and Rac proteins.

The tissue distribution of Ras-GRF messenger RNA is limited strictly to the brain, raising the likelihood that a family of these factors exist. A closely related partial sequence from mouse brain, reported by Martegani *et*

*al.*¹², was identified by functional complementation of CDC25 in yeast; although it has not yet been shown directly to be an exchange factor for Ras, it seems highly likely that it is. A larger clone of this cDNA also possesses Dbl homology (D. Lowy, personal communication). The relationship of these mammalian CDC25 homologues with the GDP-dissociation stimulator of p21-like small GTP-binding proteins (smg p21 GDS)¹³ is unclear: this exchange factor is considerably less specific, being active on Rho, Rap and Ki-Ras, but not RasH or RasN. It also requires post-translational modification of the GTPase, whereas Ras-GRF is active on the unmodified protein. The level of sequence similarity between GDS and CDC25, although detectable, is very low. ►

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An exciting prospect raised by the cloning of a mammalian Ras-specific exchange factor is that we will soon be able to understand the mechanisms by which these proteins, and hence Ras, are controlled. Nerve growth factor has been shown to stimulate Ras-exchange activity in PC12 cells¹⁴, implying that tyrosine phosphorylation is involved in some way: the nature of the connection between tyrosine kinases for growth factor

receptors and exchange factors is just one of a large number of questions that can now be addressed. Altogether, the goal of obtaining a complete map of the signalling components from growth factor to DNA-transcription factor has moved one step nearer. □

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GEOCHEMISTRY

A better mousetrap

William H. Casey

GEOCHEMISTRY would be much simpler if the Earth weren't involved. Description of the chemistry in a simple stream can easily require over a hundred thermodynamic variables, and many minerals produced by ancient fluids cannot be synthesized in the laboratory or, if synthesized, are so poorly ordered as to be useless for thermodynamic analysis. But thermodynamic analysis of mineral-fluid reactions has been made considerably easier through work by Sverjensky and Molling¹ and Sverjensky² on page 310 of this issue, who have developed a clever linear free energy relation for predicting free energies for the formation of crystalline solids and rate constants for their reaction.

Linear free energy relations (LFERs) are 'extrathermodynamic' correlations between equilibrium constants for substituted structures, or between free energies of reaction and activation energies for reactions involving substituted structures. They are indispensable in organic chemistry, where the chemical behaviour of a compound is related to the functional groups arrayed on a carbon backbone. The much-used Hammett function, for example, relates the logarithm of the ratios of rate coefficients (k) or ionization constants (K) for *meta*- or *para*-substitutions on the chemical properties of aromatic compounds:

$$\log(k/k_0) = \rho \log(K/K_0) = \rho \sigma$$

where the subscript 0 denotes benzoic acid in water, ρ is an adjustable parameter that depends on the solvent and reaction, and σ is an empirical substituent parameter for the compound of interest. Typical use of a Hammett function might be to predict the rate coefficient for hydrolysis of substituted esters. Benzoic acid esters with a methyl group would not be expected to dissociate, or react, at the same rate as an otherwise identical ester with an ethyl group. With this extrathermodynamic function, equilibrium properties, which are easy to

measure, are correlated with reaction rates, which are difficult to acquire.

Sverjensky and Molling¹ reasoned ingeniously that crystalline solids with the same structure might also be considered as substituted structures and that an LFER similar to the Hammett function could be derived for predicting thermodynamic properties. Their analogous function predicts free energies to within about 1 kilocalorie per mole, which is of the same order as the error of measurement. The correlation is necessarily threefold: one term accounts for the mineral structure; a second addresses wholly coulombic interactions using the ionic radius of the cation in the appropriate coordination; and the last term includes non-coulombic factors, such as ligand-field energies, which may be obtained by comparison with the hydration energies of the dissolved cation.

The resulting correlation will prove immensely useful in inorganic and geochemistry. Natural calcite, for example, typically contains 6–20 mole per cent MgCO_3 . Choosing an end-member component for modelling thermodynamic properties of these minerals is difficult, in part because the relevant experimental data for magnesite (MgCO_3) are scattered. The LFER allows one to sift the data for bad numbers before beginning the exercise. Even energies for hypothetical compounds can be calculated. Phosphor chemists, for example, might like to estimate the energetics of mixing of Mn_2SiO_4 and Zn_2SiO_4 to form manganiferous willemite ($(\text{Zn}, \text{Mn})_2\text{SiO}_4$) phosphor. Unfortunately, the crystalline form of the manganous end-member is tephroite (Mn_2SiO_4) that has the olivine, not the willemite, structure. One could easily estimate a free energy for formation from the LFER of Sverjensky and Molling¹.

In this issue, Sverjensky extends the method to predicting rate coefficients for dissolution of some simple crystalline compounds. The importance of this result can be easily missed — the result is

significant because some of the substituent parameters are derived from the correlation of equilibrium free energies. As such, the result links the LFER for crystalline solids even closer to better-understood functions from organic chemistry. The correlation works because dissolution rates for the chosen minerals are controlled largely by short-range forces^{3,4} and any correlation based upon bond strength would probably serve equally well. Stated differently, however, Sverjensky's LFER will undoubtedly prove useful for predicting rates of many reactions controlled by short-range forces between metals and oxygens. One might use the correlation to predict diffusion coefficients in isostructural solids; or even deformation and recovery rates in solids damaged by ion beams⁵. The beam fluence required to ruin long-range order in the mineral varies with mineral structure, but is strongly influenced by short-range forces⁵. This problem is ideally suited for such systematic examination.

Some caveats are in order. First, the correlation provides no information about a reaction mechanism. The rates of dissolution will diverge considerably from the predicted values, for example, if metals at the mineral surface are affected by electron-exchange during dissolution, whereas such complications can be easily handled with a more mechanistic approach^{3,4}. The correlation likewise provides no information about the associative or dissociative character of metal-ligand interactions at the mineral surface. The urge to interpret the empirical parameters in terms of elementary reactions at the mineral surface should therefore be strongly resisted.

Second, the correlation between bond strength and reaction rate may disappear as more complicated structures are treated than those considered in this issue. Rock-forming silicate minerals have extensively polymerized silicate structures, unlike the simple oxide and orthosilicate minerals used in the correlation. Hydrolysis of the bridging bonds in the silicate polymer probably controls the rates for these minerals and not the protonation and hydration of bonds between divalent metals and oxygens. Therefore the extreme range of rates for different minerals will probably disappear as the structures become more polymerized. (Sverjensky can hardly be blamed for choosing the simple minerals,

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as there are no comprehensive data for more complicated structures.)

Nevertheless, the LFER is a wholly empirical approach that happens to work very well in organic chemistry and promises to work well for inorganic solids. The potential power is numbing — as of 1953⁹, the Hammett function had been used to make 42,000 predictions of rate

or equilibrium constants. With this kind of number, Sverjensky's LFER will attract considerable attention from even the most jaded of experimentalists. □

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DEVELOPMENTAL BIOLOGY

How cells know their place

Helen M. Blau

How do cells specialize during development with respect to their position in the body? The general view is that gradients of morphogenetic molecules provide positional information which influences cell differentiation¹, the morphogens diffusing from a source and establishing a continuous range of concentrations. The composite of signals received by neighbouring cells differs, leading to variations in gene expression. In *Drosophila*, such positional information has been shown to play a central part in determining which end of the embryo will develop into head or hind. But in mammals there has been remarkably little evidence for such a mechanism for establishing cell diversity and pattern.

Now, however, groups led by J. R. Sanes² and N. Rosenthal³ infer that such positional signals must exist. This conclusion is based on the aberrant expression of a gene construct in the skeletal muscles of transgenic mice.

Three lines of mice were produced that carried a fusion gene in which promoter and enhancer sequences of the myosin light chain 1 (*MLC1*) gene controlled the expression of the bacterial chloramphenicol acetyltransferase (*CAT*) gene⁴. As was expected from previous transient transfection experiments⁵, the *MLC1*–*CAT* construct was expressed only in skeletal muscle, not in heart or smooth muscle or any other tissue of the mouse. Not expected was the finding that the transgene was expressed at different levels spanning two orders of magnitude in different muscles in a gradient along a rostrocaudal body axis⁶. By contrast, no difference in expression of the endogenous *MLC1* gene was observed among muscles.

At first sight, this finding might be construed as an artefact of the novel juxtaposition of two DNA sequences. The transgenic mice that developed heart and bone tumours in response to a fusion gene containing sperm-specific regulatory sequences linked to the SV40 T antigen gene⁷ provide a case in point. But a more creative and intellectually satisfying interpretation of the results,

proposed earlier by the authors⁶, is that the transgene can respond to a morphogen not sensed by the endogenous gene. According to this hypothesis, the transgene unmasks a gradient not manifested in the expression of any gene yet investigated. The positional information imparted by such a graded signal could

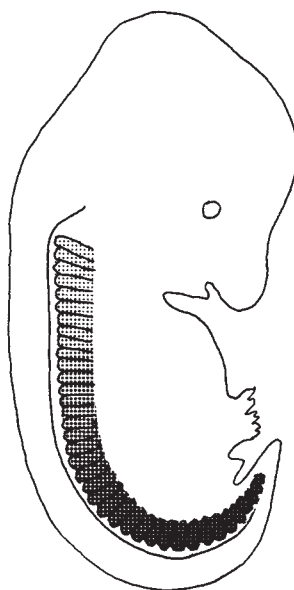


Diagram of the *CAT* expression gradient in an *MLC1*–*CAT* transgenic mouse embryo, increasing in more caudal somites (adapted from reference 3).

play a critical role in specifying cell fate during the development of the axial muscles, in particular, the intercostal muscles.

This idea is appealing. Morphogens are well known to be heavily involved in development in *Drosophila*⁸. The distribution of the products of maternal genes such as *bicoid* and *dorsal* in a concentration gradient provides a composite of signals along different embryonic axes. Both appear to be transcription factors and the *bicoid* protein contains a homeobox. The graded signals provided by these and other morphogens leads to a pattern of regions in the syncytial embryo in which specific target genes are transcribed.

In vertebrates, the best candidate for a morphogen is retinoic acid, which can alter positional values both in limb development and regeneration^{9,10}. An increase in retinoic acid concentration leads to extra limbs and digits, and even a doubling of dosage has marked effects¹¹. In vertebral development, retinoic acid seems to act by inducing the transcription of certain Hox genes that encode homeobox-containing proteins¹². The transcription factors that act on the *MLC1*–*CAT* transgene assayed in these studies remain to be identified. Members of the MyoD family of muscle-specific transcription factors are unlikely to be involved, however, because they are not found in a concentration gradient³.

The possibility that there might be a rostrocaudal gradient of gene expression in muscle was predicted nearly a decade ago¹³. Reinnervation of axial muscles implanted into the neck appeared to be determined by their previous position: caudal nerves preferentially had inputs into muscles of caudal origin and rostral nerves formed synapses on rostrally derived muscles. The data revealed a subtle (less than two-fold) but significant preference based on position. By contrast, the 100-fold difference in transgene expression observed in intercostal muscles along a rostrocaudal axis is quite robust, possibly because the mice had on average 40 copies of the transgene which amplified the effects of a subtly graded signal.

Is the gradient transient or persistent? Sanes and colleagues² show that the expression of the transgene in the adult is cell autonomous and heritable. When myoblasts were isolated from different muscles along the rostral–caudal axis, colonies expressed *CAT* activity at levels commensurate with their position of origin: highest in the most caudal and lowest in the most rostral muscles. This finding suggests that the morphogen need not be present at the adult stage. Indeed, the experiments of Rosenthal and colleagues³ indicate that the level of transgene expression is already set in the somites of the early embryo, a level inherited by the cells to which they gave rise in tissue culture (see figure). Thus, a memory, or transcriptional set point, seems to be established before the cells migrate out and reach their eventual destination in the intervertebral space.

By what molecular mechanism is the memory for the graded signal established and maintained? The memory could be fixed by heritable methylation, as in the case of X-chromosome inactivation or imprinting. Alternatively, the level of transgene expression could be determined by the protein composition in a cell at any given time, an active memory mechanism requiring continuous regulation by a network of feedback loops¹⁴.

These possibilities can be readily addressed experimentally. First, the transgene methylation pattern can be correlated with expression level in embryonic somites and adult axial muscles at different positions. To test whether expression of the transgene can be altered by a shift in composition of intracellular regulators, cultured cells from rostral and caudal positions could be fused in heterokaryons. Such experiments would determine whether an inactive transgene can be activated or an active transgene silenced by positive or negative regulators, respectively. If the transgene does exhibit position-dependent differences in methylation, this type of experiment would further address the possibility that the methylation associated with the maintenance of stable and heritable patterns of gene expression must be continuously regulated through feedback mechanisms. Perhaps even stable cell memories resulting from Lyonization or imprinting can be disrupted and lost¹⁴.

A caveat in the interpretation of the new results^{2,3} is that it is not yet clear which element within the transgene is responsible for its graded expression along the rostral-caudal axis. The expression pattern could derive from sequences within the reporter gene, not the myosin light chain regulatory sequences within the enhancer and promoter. Alternatively, expression could reflect the combinatorial nature of transcription, a unique pattern resulting from the proximity of these two particular elements in the fusion gene⁷.

That transgenic animals with a fusion gene containing regulatory sequences from the acetylcholine receptor linked to *CAT* do not exhibit a gradient of *CAT* expression in the axial muscles is reassuring⁶. But to rule out these possibilities it will be necessary to repeat the

experiment using a different reporter gene and following mutagenesis of the myosin light chain regulatory sequences. In addition, the transgene should be introduced into another mouse strain, because in a given transgenic mouse there may be either uniformity among cells in transgene methylation and expression level¹⁵ or mosaicism with cells differing in both parameters¹⁶. Moreover, as these reports attest, either pattern of methylation and expression can be due to the transgene itself or the mouse strain in which the transgene was analysed. Nonetheless, a gradient of transgene expression has not previously been reported and is likely to reflect a graded signal of some sort.

Elucidation of the nature of the regulators responsible for the cell auton-

omous memory of the gradient will require further work. The immortalized cell lines may permit identification of regulators in the signal transduction pathway by genetic complementation with appropriate complementary DNA expression libraries or by subtractive hybridization. If a sequence within the myosin light chain promoter/enhancer is found to be required for the gradient, it may prove useful in purifying the transcription factor that regulates the level of transgene expression. If not, the transgene may merely prove useful in working out the mechanisms that control penetrance and imprinting. □

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SOLAR ASTRONOMY

Darkness can illuminate

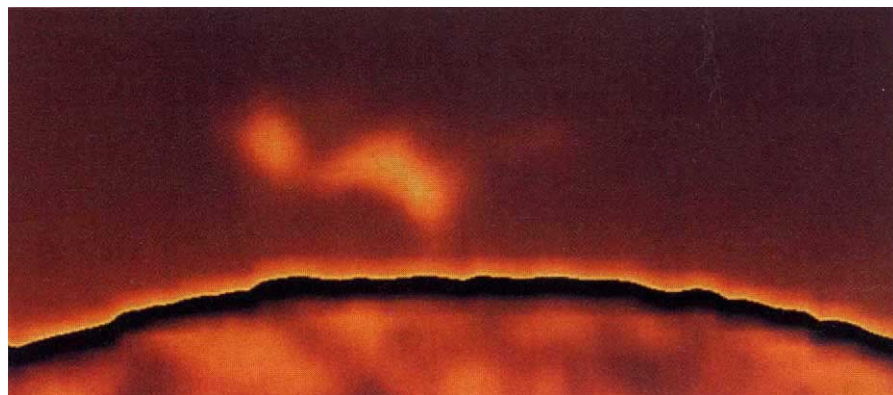
Peter Foukal

THE lucky coincidence in angular diameters of the Sun and Moon gives us spectacular eclipses, which have long been used by astronomers to study why the atmosphere of our star does not end at the edge of its photospheric disk, but actually extends out to the Earth and far beyond. This fruitful tradition of solar eclipse use continued on 11 July last year when the path of totality passed over the world's largest submillimetre-wave telescopes on Mauna Kea, enabling Lindsey *et al.* (see page 308 of this issue¹) to achieve unprecedented height resolution in the chromospheric layer of the Sun's atmosphere.

The chromosphere was first recognized as a thin annulus of red emission (the Balmer- α line of hydrogen) that appears around the photospheric rim when the Sun's glaring disk is occulted

near eclipse totality. Observations of other, relatively high excitation, lines in its spectrum first showed around the turn of the century that its plasma temperature is significantly higher than that of the photosphere. The mechanism responsible for raising this layer to these elevated temperatures has been sought in wave or electrical current dissipation, as thermal transport from the cooler photosphere below is clearly ruled out.

A further challenge is to identify how such waves might propel the elongated plasma jets, called spicules, that account for the surprising radial extent of the chromosphere. This extent far exceeds the hydrostatic-pressure scale height at chromospheric temperatures of about 8,000 K. The importance of such understanding grows as the effect of variations in the ultraviolet emission from these



A bonus, as astronomers trained their telescopes on the Sun before last year's total eclipse, was the appearance of a giant solar prominence, which Lindsey and colleagues duly imaged (for the first time) in millimetre-wave radiation. The resulting picture, from the James Clerk Maxwell Telescope, on Mauna Kea, shows the prominence extending up to 75,000 km from the Sun's surface. The prominence consists of relatively dense plasma (10^{10} ions cm^{-3}) at a temperature of 5,000 K, similar to that of the solar disk. (Courtesy C. Lindsey.)

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plasma layers on stratospheric ozone becomes increasingly apparent.

The millimetre-wave observations by Lindsey *et al.*, and by three other groups during the same eclipse²⁻⁴, provide new insights on the dynamics of this layer and its role in the mass and energy balance of the tenuous corona and solar wind. The millimetre waves originate by hydrogen free-free emission in the chromospheric plasmas. Their high optical depth enables the observers to relate fluxes linearly to the plasma temperature through the Rayleigh-Jeans approximation to the Planck function. This is very helpful in these heterogeneous layers which are sufficiently opaque to optical and ultraviolet radiation to make calculations of radiative transfer messy, and yet are not opaque enough to equalize entirely the plasma and radiation temperatures. These conditions preclude the use of many standard optical and ultraviolet spectral diagnostics commonly employed to measure plasma temperature and density in the optically thin coronal plasmas.

Resolution

Even with the largest millimetre-wave dishes diffraction limits the angular resolution of observations to values in excess of 20 arcseconds. Such coarse resolution is of limited use for measurements of structure in the chromospheric layer whose extreme radial extent of about 15,000 km subtends only 20 arcseconds at the Earth. But during an eclipse, the difference between two successive measurements made as the Moon moves across the face of the Sun represents the flux from the narrow strip exposed or hidden in the intervening time. This way, sub-arcsecond resolution can be achieved even with a 1-second sampling interval, given the Moon's speed across the solar disk of about 0.5 arcsecond per second.

The measurement is most precise when the bright disk is almost entirely occulted by the Moon, as occurred in the observations performed around totality a year ago from Mauna Kea by Lindsey *et al.*¹ at 1.3 mm, and by Ewell *et al.*² at 0.85 mm. However, the technique was also applied with interesting results under partial eclipse on the same day using millimetre-wave interferometers in California, by White and Kundu³ at 3.5 mm and by Belkora *et al.*⁴ at 3 mm.

The measurements indicate that the Sun's disk extends some 5-8 arcseconds beyond its photospheric edge, with the largest extensions seen at the longest wavelengths, as expected from the frequency dependence of black-body emission and free-free opacity. This extension agrees well with the heights of the spicule jets, which are observed in monochromatic Balmer- α images⁵ to

heights in excess of 7,500 km.

It is interesting that Lindsey *et al.* report spicule temperatures of only 6,000-8,000 K (thus not much hotter than the photosphere). This implies that while the mechanism that propels these plasma columns can raise them to heights well beyond those expected from their ballistic trajectory, it does so with surprisingly little dissipation of energy. It was already difficult to understand how waves of sufficient amplitude to lift spicules (presumably generated by turbulence in the photosphere) could survive the powerful dissipation that must sap their power in the intervening layers⁶. The low spicular temperature exacerbates this problem.

Absence

But the most interesting result is in what the millimetre-wave observers do not see. Optical observations do not determine what happens to the cool plasma in spicules beyond their maximum height as observed in Balmer- α . The subsequent fate of this material is an important issue, because the globally integrated spicular mass flux exceeds that in the solar wind by two orders of magnitude. It could be that the spicules simply stop radiating Balmer- α emission because they are heated sufficiently, to 10⁵-10⁶ K, and become part of the corona. But the power required to heat them to such temperatures would far exceed what is needed to lift them against solar gravity and to accelerate them to their observed velocities.

The absence of any signal in the millimetre data above 7,500 km provides interesting evidence that there is no such mass of hot, dense material beyond the tops of the Balmer- α spicules. Certainly, any spicular material heated to 10⁵ K, with a consequent increase in ionization, would be conspicuous in millimetre radiation (and also in the extreme ultraviolet⁷). In fact, the absence of any signal seems also to dispose of the view⁸ that spicular material lifted high above the chromosphere could fall back and heat this important layer of the sun's atmosphere, simply by release of potential energy. □

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Breathless hush

ALL the best chemical processes use continuous flow: reagents go in at one end and products come out at the other. Air-breathing engines follow the same principle, as does the transpiration of plants, the breathing of fish and our own digestive systems. One strange biological exception is the breathing of animals, including ourselves. Air is alternately sucked into, and blown out of, the same aperture. This is extremely inefficient. Most of the oxygen we breathe in is simply breathed out again unused (which is why 'kiss-of-life' resuscitation works). Daedalus is now devising continuous-breathing technology.

His first idea was to pass a pipe in through one nostril and down into the lungs, and to pump air down it. Fresh air would thus be introduced into the lungs continuously, and used air would emerge from the other nostril. But even a pipe carefully chosen to match the compliance and elasticity of human tissue would probably feel foreign and most unpleasant. So he now proposes a completely non-invasive technique.

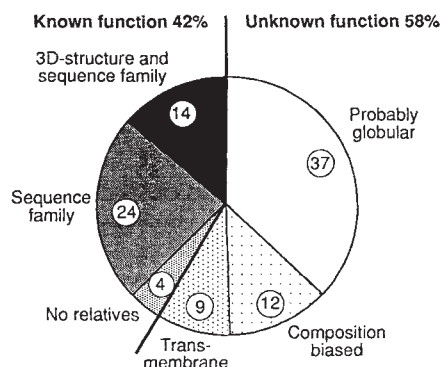
It is based on the fact that oxygen is paramagnetic, and can therefore be pumped along by the moving magnetic field of a linear motor. DREADCO technicians are devising a sort of high-collared strait-jacket wound with multiple wire coils. Energized with the correct relative phasings, the coils create a magnetic motor-field which pumps oxygen strongly into the wearer. The molecules are impelled into his nostrils, and right down to the deepest recesses of his lungs. Carbon dioxide, however, is feebly diamagnetic and is displaced the other way. The result is a two way diffusion process in the wearer's air passages. Oxygen molecules flow steadily in, threading their way past carbon dioxide molecules on their way out. Nitrogen molecules are little affected.

The wearer can therefore safely stop breathing. His 'Respirovest' will do it for him, in a continuous-flow process far more efficient than the old reciprocating bellows mechanism. He will enjoy a wonderful bodily calm, breathing only in order to talk — and even then it should be possible to allow the Respirovest to fill his lungs passively with oxygen, and only have to breathe out. The Respirovest will also have serious uses. People with cracked ribs or lung diseases will welcome its completely static and highly efficient action. Firemen and rescue workers will use it too. Even the thickest smoke and fumes usually contain a lot of oxygen. By selectively pumping oxygen into their lungs while repelling all other molecules, the Respirovest will act as a wonderful gas mask. David Jones

What's in a genome?

SIR — We have taken up the challenge to elucidate the function of the 182 predicted protein products derived from the complete DNA sequence of yeast chromosome III, a result of the European yeast genome project (S. Oliver *et al.* *Nature* **357**, 38–46; 1992). These authors report that some functional information is available for about 57 of these proteins, either determined by experiment or deduced by similarity searches in sequence databases.

We have identified the probable function of 17 additional protein products, using a combination of low-stringency sequence database searches, various



Yeast chromosome III proteins. Information accumulated to date by all methods, experimental and theoretical. Information content increases counterclockwise. The principal diversion is between known and unknown biological function. Numbers in per cent. Composition bias indicates unusual amino-acid composition untypical of globular proteins, for example, in coiled coils. The categories are approximate, but give an impression of the current state of the art.

ways of assessing significance, multiple sequence alignment, pattern searches and incorporation of prior knowledge about protein and domain families. The most interesting of these include a DNA polymerase of type X previously found only in mammalia, a new regulatory domain common to eukaryotes and prokaryotes (PILB), a methyltransferase, an acetolactate synthase and a GAL4-type transcriptional activator (see table). In addition, we find that 25 of the chromosome III proteins have homologues of known three-dimensional structure. Taken together, as many as 42% of all proteins of yeast chromosome III have a known or probable function and 13% have an indirectly known three-dimensional structure. Of the remaining 58%, about one-third have putative transmembrane segments (see figure).

Extrapolating from chromosome III to the entire yeast genome, we can expect that the white, uncharted areas cover only about half of the protein function map but as much as six-sevenths of the protein structure map. As genome projects provide more and more raw sequence data, informatics methods such as those used here can cover much ground, but efficient experimental methods are needed to determine the structure and function of proteins without similarity to known families.

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SIMILARITY OF SELECTED CHROMOSOME III PRODUCTS TO OTHER PROTEINS

ORF	Length	Family	Closest	%id/len
YCL9c	309	Prokaryotic acetolactate synthases, small subunit	ILVH_ECOLI	36/170
YCL19w	1,347	Transposon B gene family, related to pol genes	COPI1_DROME	18/505
			POLX_TOBAC	20/393
YCL20w	438	Transposon A gene family	YTY1_YEAST	49/439
YCL33c	168	Repressor of pilin promoter	PILB_NEIGO	33/110
YCL75w	146	Pol-like protein	S00954(P)	40/74
YCR14c	582	Type X DNA polymerases	DPOB_RAT	26/393
YCR23c	611	Tetracycline resistance proteins	TCR1_ECOLI	28/150
YVR26c	743	Mammalian PC1 plasma cell membrane protein phosphodiesterase family	PC1_HUMAN	38/129
			PPD1_BOVIN	38/66
YCR32w	2,167	Hypothetical protein related to C-terminal 'CDC4'-like human fragment	HSCDC4A(E)	49/316
YCR36w	333	Ribokinase (other prokaryotic sugar kinases)	RBSK_ECOLI	38/96
YCR47c	275	ApoMet-methyltransferases	GLMT_RAT	26/301
YCR64c	136	Carboxypeptidases N	CBP8_HUMAN	27/88
		dipeptidyl-peptidase IV	DPP_LACLA	25/105
YCR69w	170	Peptidyl-prolyl-cis-trans isomerases	CYPH_CANAL	37/122
(YCR70w)*				
YCR72c	541	G-protein beta subunits	PR04_YEAST	23/278
			TUP1_YEAST	32/110
YCR98c	518	Sugar transporter/symporter	A40260(P)	25/179
YCR104w	124	Glucose repressor/cold shock inducible	SRP1_YEAST	27/115
			SCTIPI(E)	27/99
YCR106w	832	Gal4-like DNA/Zn binding domain	CYP1_YEAST	45/47
			GAL4_YEAST	19/168

ORF, predicted open reading frame (Oliver *et al.*, 1992); Family, functional protein family; Closest, sequence database identifier of the closest relative(s) from SWISS-PROT (default), PIR (P) or EMBL (E); %id/len, per cent amino-acid identity/length of the alignment.

*When the two adjacent ORFs YCR69w and YCR70w are cut and fused, correcting a possible frameshift sequencing error, they together represent a single member of the proline *cis-trans* isomerase family.

Pyroelectric X-ray generator

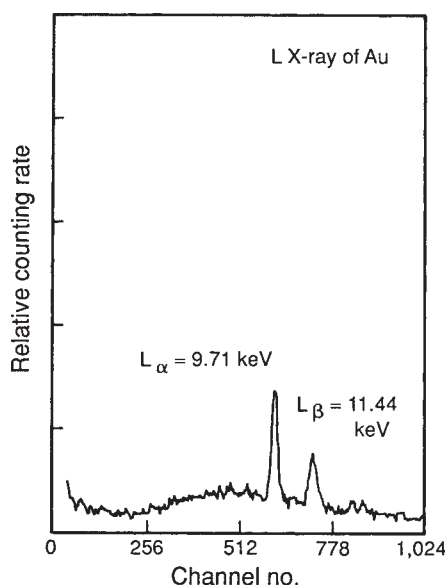
SIR — Pyroelectricity is a phenomenon analogous to the more familiar piezoelectricity, a transient voltage produced in response to a change in temperature rather than to a physical stress. Some pyroelectric crystals will also emit electrons^{1–7}; electron energies up to 10⁵ eV and electric fields as high as 10⁶ V cm⁻¹ have been obtained in single LiNbO₃ crystals. By placing a gold foil close to a pyroelectric crystal of CsNO₃, we have made a small X-ray generator which creates photons of about 20 keV.

When CsNO₃ is cooled from approximately 300 to 77 K, it produces an electric dipole, which can be maintained at constant temperature because the relaxation time is long⁸. During the cool-down, electrons are ejected from the negative end of the dipole. As the temperature is raised from 77 to about 150 K the dipole begins ejecting electron clusters in bursts from the positive end of the dipole. On warming, electron ejection starts and ends within a specific narrow temperature range for each crystal when the ambient vacuum and rate of change of the temperature is constant.

The sign of the ejected particles was determined to be negative by their deflection in magnetic and electric fields. The particles were detected with a silicon charged particle radiation detector and appeared to have high energy, in the MeV range. However, the beam of particles was attenuated to zero with 1.6 mg cm⁻² mylar between the crystal and the detector.

When a cluster of electrons with a spread in time less than the resolving time of the system arrives at the detector, the output will reflect the total energy deposited in the detector by the electrons. Therefore, a closely packed cluster of about 15-keV electrons and a 5-MeV α -particle may appear to deposit the same amount of energy in the detector. Using α -emitting sources to calibrate the system, clusters of electrons with an apparent total energy as high as 10 MeV are observed.

In the figure we show the characteristic L X-rays of gold that are produced when a gold foil is adjacent to the positive end of a CsNO₃ crystal. A Si(Li) X-ray spectrometer with a 160-eV resolution was used to make this spectrum. These X-rays are produced as the temperature of the crystal is raised from 77 to 300 K and the foil is bombarded with ejected electrons. Fluorescent L X-rays result from vacancies produced in the L shell of Au atoms, which requires an energy of 14.3 keV. However, the K X-rays in Ag, requiring 25.5 keV, were



Spectrum of Au L X-ray detected with a Si(Li) X-ray spectrometer.

not produced, thus providing an upper limit for the energy of the ejected electrons.

The X-ray generator is a small vacuum cryostat with a liquid nitrogen reservoir, a thin window and a heater/cold finger. One end of the heater/cold finger is connected to a liquid nitrogen reservoir and the other faces the window. An ambient vacuum of 5×10^{-5} torr is sufficient. The crystal is placed on the end of the heater/cold finger with the positive end approximately perpendicular to the target and parallel to the window of the cryostat. A simple spring clip holds the crystal in place. The heater is a 47- Ω wire-wound resistor inside a brass tube that is the cold finger. The temperature of the cold finger is monitored with a thermocouple and the cryostat window is 4.5 mg cm⁻² aluminium.

A typical CsNO₃ crystal grown in an aqueous solution is $0.2 \times 0.3 \times 0.8$ cm and is very fragile. When these crystals are cooled for the first time, they fracture and fragment. To prevent separation after fracturing, crystals are covered with a thin layer of epoxy. The ends may or may not be covered, as a thin layer of epoxy on the ends has little effect on the production of X-rays. The electrons accelerated by the crystal come from the ionization of molecules of the residual gases in the system.

The polarity of each crystal is determined by suspending it in liquid nitrogen in an electric field of known polarity and observing the alignment of the crystal with the electric field as it cools. If any part of the crystal is not covered with epoxy, all water must be removed immediately following this procedure.

To produce X-rays the heater is turned on and off, cycling the crystal between approximately 77 and 273 K.

The cycle time is about 5 min, with about 1 min of X-ray production. With a thin window NaI(Tl) detector and a gold foil 0.5 cm from the crystal, 150,000 photons are detected in less than 1 min. No apparent reduction of X-rays is observed after repeated temperature cycles or if the crystal is maintained for long periods at either 77 or 300 K.

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APC mutations

SIR — The function of multimeric proteins, or multiprotein aggregates, can be altered by inhibitory polypeptides produced through the action of dominant negative mutations¹. The discovery of loci involved in colon cancer² which are dominant for polyp formation prompted Bourne to propose that these mutations exert their phenotype by this mechanism³. However, in a more recent paper, Groden *et al.*⁴ argued that the nature of the observed mutational events in these patients with adenomatous polyposis coli (APC), which were found to be nonsense mutations and frameshifts, is inconsistent with such a model (the observed mutational events are incompatible with a dominant negative phenotype).

The consequence of such mutations, however, need not only be the inactivation of the gene and its protein product; through the process of translational reinitiation they can also alter the function of the product. In bacteria⁵, nonsense and frameshift events that create

or activate barriers to translation result in the production of dominant restart polypeptides in genes or proteins that exhibit an appropriate structure and function. This process provides a ready explanation for the APC results, namely, the carboxy-terminal fragments so generated by reinitiated translation interfere with the normal functioning of APC and/or MCC (mutated in colon cancer) protein complexes. Indeed, such a phenomenon may be particularly important in the alteration of the function of proteins insensitive to amino-acid substitution. Such functions would be sensitive to various mutational events that alter the reading frame (single-base frameshifts, deletions, duplications and rearrangements) while providing only a limited target for missense mutations. These mutational events have been neglected of late because of the predominance of single-base substitutions in the oncogene/suppressor gene systems so far characterized.

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Time machine from dust

SIR — Allen and Simon¹ explain how a pair of cosmic strings, if they exist, could form a time machine. However, there are several other space-time metrics of general relativity which have this property, some of them quite simple.

Probably the simplest is the one discovered long ago by van Stockum^{2,3}. This refers to the gravitational field outside an infinitely long circular cylinder in steady, rigid rotation. The cylinder is made of dust, that is, matter whose constituent parts have no interaction except their mutual gravitation. The properties of the system are governed by the mass per unit length, m . When m is not very large the gravitational field outside the matter is just that of a static infinite cylinder. This is surprising because it means that, contrary to expectations from Mach's principle, the rotating matter does not drag the exterior space-time with it.

When m is large (in fact, greater than 2×10^{27} g cm⁻¹), the rotating matter does drag the exterior space-time, and closed time-like lines exist outside the cylinder. Therefore an observer, using rockets, could travel into his past: the

Scientific Correspondence

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rotating cylinder constitutes a time machine.

Of course, this time machine, like that described in ref. 1, requires an infinitely long object, and it may be that the properties of a very (but not infinitely) long cylinder would be different. The particularly intriguing feature of van Stockum's time machine, in contrast with others in general relativity, is that it is made of such homely material, namely dust.

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Earliest *Homo* debate

SIR — The report by Hill *et al.*¹ of the "earliest" *Homo* from near Lake Baringo in Kenya betrays a common misconception about the relationship between fossils, isotope dates and the strata from which they are recovered. The widespread discussion²⁻⁴ of the finding of Hill *et al.* demonstrates the need for some critical interpretation of the actual data.

The fossil specimen, KNM-BC1, was recovered from the Chemeron formation in 1965 (ref. 5). Although no isotope dates were then available, its stratigraphic position was documented, and this allowed Hill *et al.*¹ to attempt to assign it an age. The local geological context allowed its source to be ascertained with "little doubt"⁵, and the source was "about 8 ft from the base of the Upper Fish Beds"⁵. Hill's team returned to the locality (JM85) to collect dating materials from close proximity to, and in sequence with, the hominid level. This is fairly typical of important hominid sites, where geologists attempt at a much later date to control the age of significant finds.

The next step is to produce isotope ages, both accurate and reasonably precise, with which to control the age of the fossil. In this respect the report by Hill *et al.*¹ is exemplary. Replicate ⁴⁰Ar/³⁹Ar dates on two samples of pumice from a tuff, lying about 2.5 m below the stratum from which the hominid was recovered, each produced weighted means of 2.45±0.02 megayears (Myr), and an isochron of 2.43±0.02 Myr. The dating of this unit appears to be both accurate and precise, and is well supported by the data.

The problem with the age of the fossil comes from a failure critically to evaluate the relationship of the dated stratum

to the hominid-bearing level. The date of 2.45 Myr does not apply to the fossil, but rather to a level 2.5 m below its resting place. Were a modern hominid to go to JM85 and recline on the dated tuff, he/she would overlie the 2.45-Myr level, but would not be 2.45 Myr in age. Similarly, the fact that the fossil was stratigraphically above the dated tuff does not make it 2.45 Myr old.

Although the case for an age of 2.4 Myr for the fossil is unfounded given the data presented, this does not necessarily mean that it is not close to that age. It simply means that the fossil has only been documented to be younger than 2.45 Myr. Units within the Chemeron formation range as young as 1.6 Myr¹, and as no stratigraphic sections are provided for the site, its relationship to these younger strata is unclear. This does not lend credence to the claim that KNM-BC1 is the "earliest" *Homo*. The figure required to support that assertion is a date on a unit which stratigraphically overlies the hominid level. The statement in ref. 1 that "(p)reliminary age results from tuffaceous units higher in the sequence (A. H., S. W. and A. D., manuscript in preparation) are consistent with these results, and support an age of about 2.4 Myr for the hominid" is misleading. It was misinterpreted by Gibbons³, who commented that the authors "used a new technique . . . on volcanic deposits above and below" the fossil. This implies that dates were determined for both levels. If the data "in preparation" provide the conclusive control on the fossil, and show it to be 2.4 Myr in age, then they should have been included. If such results are only anticipated to support the age, then they are irrelevant.

The case for the "earliest" *Homo* at Lake Baringo is unsupported. Careful consideration of isotope age data, stratigraphic context and the limits of interpretation⁶ are necessary in any discussion of such evidence critical to understanding the ancestry of man.

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SIR — A fragment from the right temporal bone of a fossil hominoid (KNM-BC1) from the Chemeron formation of Kenya was recently dated by Hill *et al.*¹ to approximately 2.4 Myr and attributed to the genus *Homo*. If the date and generic attribution are correct, the fossil is important because it represents the earliest securely known fossil from our own genus. The attribution of the temporal fragment rests on the assertion that it manifests two features unique to *Homo*: a sharp petrous crest, and a

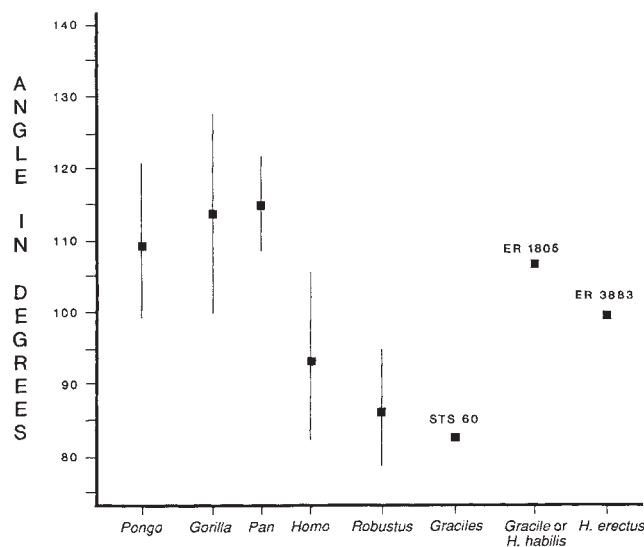
medially positioned mandibular fossa. But the observations regarding both features were offered without supporting measurements.

Hill *et al.* overlooked an earlier observation⁷ that the angle of the petrous crest (just lateral to the posterior margin of the internal acoustic meatus) is sharp in the robust australopithecine OH-5 (*Zinjanthropus*), as is the case for modern humans. However, that earlier study did not provide specific angles for the petrous crests of OH-5 or modern humans, possibly because of the difficulty of measuring this angle in intact (closed) skulls. We have now overcome this problem by measuring the petrous crest angle reproduced on the right sides of endocasts representing *Pan troglodytes* ($n = 5$), *Gorilla gorilla* ($n = 4$), *Pongo pygmaeus* ($n = 6$) and *H. sapiens* ($n = 4$). In addition, we measured the petrous crest angle from endocasts of five fossil hominids including OH-5 and SK-1585 (robust australopithecines), STS-60 (a gracile australopithecine), KNM-ER 1805 (either *Australopithecus* or early *Homo*) and KNM-ER 3883 (*Homo erectus*).

Endocasts, which accurately reflect the external shape of the brain (and other morphology), were prepared from ape and human skulls using liquid latex⁸. Each hollow endocast was filled with plaster, taking care to preserve its original shape. To measure the angle of the petrous crest, a second cast was prepared from the right petrous temporal region (on the exterior of each endocast) using alginate casting material. The resulting casts clearly reflected the bony morphology of the entire petrous region of the internal skull.

The angle of the petrous crest was measured before the alginate cast dried and possibly changed shape. In order consistently to measure the same part of the petrous crest, we recorded its angle at a location just lateral to the posterior margin of the internal acoustic meatus, using an apparatus consisting of two straight edges joined at a rotatable pivot. After the edges were rotated so that they conformed to the angle of the petrous crest, the apparatus was removed and the angle read with a protractor. This procedure was repeated four times on each cast, and the mean of the measurements taken as the angle for that cast. A remeasurement analysis revealed that the measurements for each specimen departed from that specimen's mean by an average of less than 1.7°. When this error is expressed as a fraction of the mean for each specimen, and the data are then averaged across the 24 specimens, the mean remeasurement error is less than 1.6%.

Despite our small sample sizes, several conclusions may be drawn from our



Petrous crest angles in degrees. Error bars 1 s.d. above and below the mean are included for samples having more than one observation. All measurements from the right hemisphere except for STS-60. The mean angles are 110° for *Pongo*, 114° for *Gorilla*, 115° for *Pan* and 94° for *H. sapiens*. Robust australopithecines include OH-5 (95°) and SK-1585 (79°). Other fossils include STS-60 (83°), ER-1805 (107°) and ER-3883 (100°).

results: (1) The angle of the petrous crest was smaller (sharper) in the four humans than in the 15 pongids ($t(17) = 2.86$, $P < 0.02$, two-tailed), although the range for humans overlaps to some degree with those of all three great apes. (2) Tobias' suggestion⁷ that the angle of the petrous crest of OH-5 is like that of modern humans is supported by our measurements (OH-5 = 95°; *H. sapiens* = mean of 94°). (3) There appears to be extensive overlap between the range of angles of the petrous crest for *H. sapiens* and that for a variety of fossil hominids. (Of the five fossil hominids that we measured, only ER-1805 fell slightly above the human range.) (4) As the figure clearly shows (and as *t*-tests confirm), a sharp angle of the petrous crest fails to separate extant and fossil *Homo* from australopithecines. Because the *Homo* and australopithecine petrous crest angles are so similar, no matter what the angle of the petrous crest of the Chemeron temporal or any other fossil may be, that angle cannot be used to attribute the fossil to the genus *Homo*.

A medially positioned mandibular fossa is the second feature that theoretically sorts the Chemeron fossil exclusively with *Homo*. In an earlier study of the Chemeron fossil, Tobias compared measurements of the length, breadth and depth of the mandibular fossa in various hominids and pongids⁹. He found that the length and breadth of the Chemeron fossa fell within the australopithecine range, whereas its absolute depth fell between those for australopithecines and *H. erectus*, but nearer that for the former, leading him to conclude that the exact taxonomic affinities of the specimen could not be determined from these features. The reappraisal by Hill *et al.*¹ provides no measurements to alter this conclusion.

Since we submitted this Scientific Correspondence, Andrew Hill kindly lent us

a copy of the Chemeron temporal bone, from which we measured an angle of the petrous crest of approximately 90°.

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HILL ET AL. REPLY — The general stratigraphic point that Feibel makes about radiometric dates and hominid fossils is important but rudimentary; naturally we were aware of it as we reported in our paper¹. "... Preliminary age results from tuffaceous units higher in the sequence ... support an age of about 2.4 Myr for the hominid ..." By our statement, we meant that preliminary age results from tuffaceous units higher in the sequence support an age of about 2.4 Myr for the hominid. Our paper reported the initial phase of a continuing investigation. We had justifiable confidence in our preliminary ages on overlying strata, but felt it best to finalize these data and add more information for a comprehensive geochronological treatment of this fossil site.

For the purposes of this reply we can state that single crystal, laser-fusion ⁴⁰Ar/³⁹Ar age determinations from a tuffaceous unit occurring 4.7 m directly above the inferred level of the hominid produced a date of 2.38±0.01 Myr. Another stratigraphically higher tuff from a nearby locality, estimated by correlation of sections to be about 10 m above the hominid horizon, produced an age of 2.35±0.02 Myr. Relevant analytical details are contained in a manuscript about to be submitted to the *Journal of Human Evolution*. These dates firmly support an age of about 2.4 Myr for the hominid.

Falk and Baker's measurements of

hominid petrous crest angles have some interest, but are irrelevant to our analysis of KNM-BC1. A closer reading of our paper¹ shows that we were not concerned with the angle of the petrous crest, but with the morphology of the edge. As we reported, the edge of the petrous crest is sharp, along the surface where the tentorium cerebelli attaches to the petrous. Wood in his News and Views article² noted metric aspects of petrous temporal orientation that we intentionally did not include in our assessment of KNM-BC1. The acuteness or obtuseness of the angle of the petrous crest discussed by Falk and Baker has nothing to do with our analysis.

The second part of Falk and Baker's note purports to address the position of the temporomandibular joint fossa, but instead discusses temporomandibular joint fossa size. Falk and Baker accuse us of a lack of metric rigour without contributing any new metric observations, but say we should accept the conclusions Tobias reached on the basis of a quite small sample of early hominid crania available to him nearly a quarter of a century ago⁷. We have studied the original specimens of all published East and South African crania, and find tremendous variation in temporomandibular joint size. Moreover, the topographic anatomy of the joint is far more useful in taxonomic assessment than simple measurements of its length and breadth. We conclusively showed that the temporomandibular joint fossa of the Chemeron temporal bone is medially placed below the middle cranial fossa, as it is in *Homo*. None of Falk and Baker's comments leads us to modify our conclusions.

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The cold war remembered

Frank Close

Cold Fusion: The Scientific Fiasco of the Century. By John R. Huizenga. University of Rochester Press: 1992. Pp. 259. \$45, £29.50. Distributed by Boydell and Brewer in the United Kingdom.

RESEARCH into nuclear fusion has traditionally been regarded as a branch of physics, a 'big science' enterprise demanding vast resources. So there was surprise when, on 23 March 1989, two chemists, Martin Fleischmann and Stanley Pons at the University of Utah, claimed to have harnessed fusion in a test tube of heavy water at room temperature. Within weeks most scientists had satisfied themselves that Fleischmann and Pons's claims were exaggerated at best and seriously flawed; nonetheless, diehards continue to insist that a hitherto unknown nuclear process has been discovered, which hides all presence of itself except for the heat that Fleischmann and Pons were the first to claim. In desperation, the lobbyists are accusing the US Department of Energy (DOE) of conducting a "war against cold fusion" and claim that DOE officials, who rely on John Huizenga for advice, are being misled.

Huizenga was co-chairman (with Norman Ramsey) of a DOE panel of 22 highly respected chemists, physicists and others that investigated the cold-fusion claims in 1989. Members of the department's laboratories reported to the panel weekly; the panel members visited other institutions that had claimed to have found evidence for cold fusion and they also attended meetings on the subject. They produced a report that was unfavourable to cold fusion in late 1989 and Huizenga's book is, in part, drawn from his experiences during that period. Huizenga has continued to follow the field and attend meetings; he still believes in the non-existence of watts of power generated by cold nuclear fusion. Here he gives the most complete evaluation of the science of cold fusion so far. Furthermore, he makes abundantly clear why the claims for useful nuclear power from 'cold fusion' are groundless and raises disturbing questions about the ethics underlying some of those claims. As a distinguished nuclear chemist he is uniquely qualified to evaluate the field. Cool, dispassionate scientists and policy-makers will receive his book, I trust, with the respect it deserves.

Much of what Huizenga writes is already known but has rarely been described in such detail. An eye-opener to me, at least, is his story of the helium episode. After the production of most neutrons, tritium and gamma rays had

been eliminated in the Utah experiment, there arose a claim that fusion was producing helium-4. Pons announced this finding on television, and Cheves Walling and John Simons in his department wrote a paper on the hypothesis, citing Pons's 'discovery' as a "private communication". Eventually Pons backed off from the claim, but Walling and Simons published the paper even after it was clear that the helium findings were flawed. Huizenga comments: "The ^4He data, if true, would have been revolutionary . . . however, these data were completely erroneous and no retraction has appeared". Walling and Simons did not add any note to warn readers that the helium data were erroneous, because "apparently [they] felt it was Pons' responsibility to set the record straight". This cavalier and teasing appearance and disappearance of evidence typifies the cold-fusion saga, making it hard to have a sensible discussion about the claims. But the episode pales compared with what happened next.

In mid-1989, after much pressure, the Utah cells were tested for helium in an external 'double blind' test. A set of palladium rods, some used in cold-fusion experiments, together with control samples, were dispersed to six laboratories for analysis. Pons supplied some of the rods and his subsequent analysis makes interesting reading. The scientist responsible for overseeing the test "found to his great surprise that Pons had not given him all of the (agreed) informa-

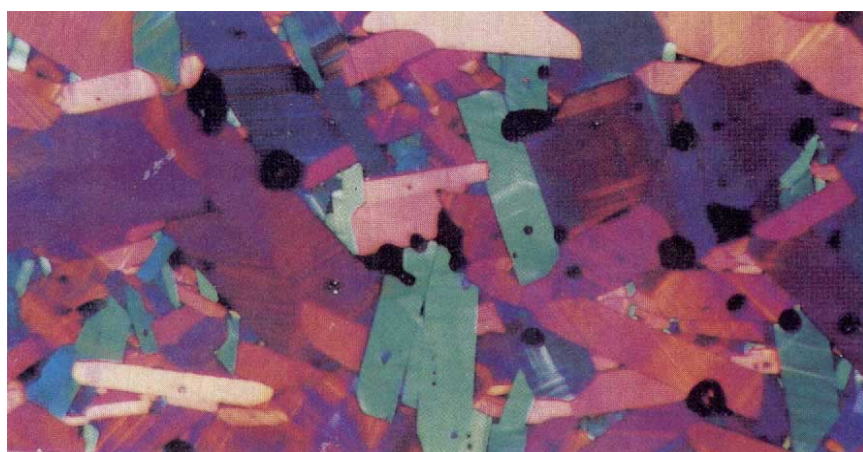
tion" and although the overseer "kept faithfully [to] his part of the bargain [t]he same cannot be said . . . for others". On page 225 Huizenga states more explicitly that Pons "renege[d]" on the agreement. Pons's attorney then accused the overseer of "leaking the results of the tests to [the] DOE panel". Huizenga states categorically that this is untrue and makes the accusation that the "two University of Utah chemists orchestrate[d]" the episode.

And there is much more like this. Huizenga tackles the science and also the ethics. He comments that "many instances occurred . . . where information was withheld or falsified", starting with the press conference. He says that the initial announcement by Fleischmann and Pons of watts of heat per single watt of electricity consumed was "inconsistent with that reported in their paper" and that "as facts became fully known the initial claims were withdrawn or severely scaled back".

The book will be immensely useful to anyone researching the history of the cold-fusion episode, so it is a pity that the text is not more fully documented. In particular, there is one important claim that I believe to be incorrect and needs further investigation.

Fleischmann spoke to a private group at Harwell on 28 March 1989, five days after the press conference and before any general scientific presentation of the Utah data: Huizenga claims that "by this time preprints of their forthcoming article in the *Journal of Electroanalytical Chemistry* had been distributed". This differs from my claim that copies of the preprint were made public only on and after 30 March.

But at the meeting on 28 March, Fleischmann was told that data on a purported gamma ray that he had displayed in a figure had nothing to do with fusion. My thesis has always been that



View of $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ superconducting oxide with crossed polarizer and analyser ($\times 250$). From *Images of Materials* by D. B. Williams, A. R. Pelton and R. Gronsky, a guide to microscopy techniques. Published by Oxford University Press, £60.

these data had already been sent to two journals but were not yet publicly known. By the time the preprints became public on 30 March, Pons had altered the numbers in the figure with the result that the data now appeared to be bona fide evidence for fusion. The world then received this 'evidence' unaware of its true origins.

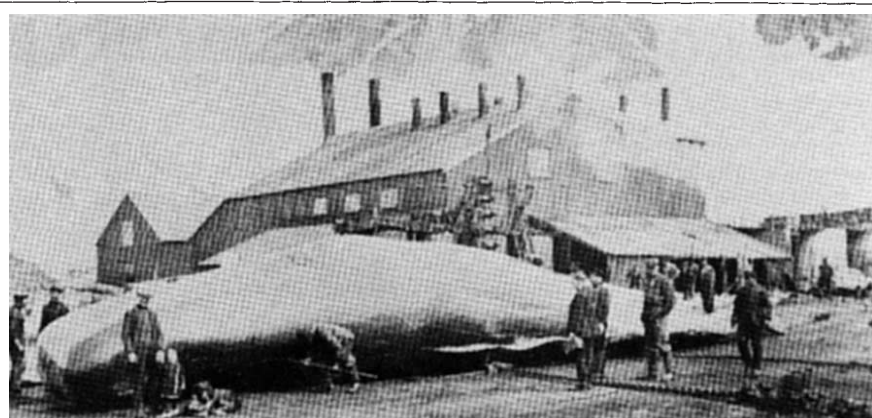
Had the real data been made public at the time of the original announcement, then the cold fusion 'fiasco' would probably never have been. So these dates are important in determining what was known to whom during the critical week after the press conference and leading up to publication of the first supporting paper. In this regard, historians may be intrigued by the claim in the chronology (Appendix 3) that Edward Teller was sent a preprint on 24 March. Huizenga documents the subsequent machinations about these particular data and comments (page 139) that "further investigation of Fleischmann and Pons' handling of the mobile gamma-ray signal peak is merited".

Huizenga draws morals about how scientific scientists should handle their publications and, if errors are found, withdrawals. He pleads that at a time where "carelessness and fraud in research are getting more public attention, experiments . . . and results [must be] honestly reported". Surely nobody would disagree, but how well prepared is the scientific community at large to respond when more than just mistakes are made? Is it really satisfactory that the policing of ethics seems to be of more concern to *The New York Times*, the National Institutes of Health and editorial columns than to scientific institutions?

Huizenga unequivocally states his opinions: the final chapter, entitled "Lessons", should be compulsory reading for anyone concerned about the conduct of science. Commenting on the hundreds of millions of dollars of research time and resources that were taken up in showing that there is no convincing evidence for cold fusion as a source of nuclear power, he notes that "much of this would not have been necessary had normal scientific procedures been followed". This should be taken on board by all scientific institutions.

And in future, how can the media, industry and general public make an informed judgement? Huizenga's cogent advice: "whenever the inability of qualified scientists to repeat an experiment is met by an onslaught of ad hoc excuses: Beware". □

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A sperm whale on the flensing plan at Grytviken (1916). Permanent settlement of South Georgia began in 1904 with the foundation of this whaling station. Photograph from *The Island of South Georgia* by R. Headland (Cambridge University Press, £19.95, \$39.95 (pbk)).

Being prepared

J. G. Shepherd

Climate Variability, Climate Change and Fisheries. Edited by Michael H. Glantz. Cambridge University Press: 1992. Pp. 450. £45, \$69.95.

THERE can be little doubt that fish stocks respond to climate change, and that the biological and economic consequences of global warming for fisheries are likely to be considerable. Whether or not science can say anything useful about these changes before they happen is another question. With a few exceptions, we cannot even be sure of the direction of the likely changes, let alone their magnitudes. The problems are difficult because of the great complexity and volatility of marine ecosystems. Trophic status in the sea is heavily dependent on size: individuals of a species may progress from being eggs through to herbivores and several varieties of carnivore in a single season, and become cannibals by the next. Also, the continued viability of a population may depend on a specific configuration of currents to prevent the dispersal of the young or to bring them safe to suitable nursery grounds. In few cases have these mechanisms been fully described, and our ability to predict from an understanding of the processes is thus limited.

One must therefore fall back on less satisfactory methods of prediction. This book is the product of a workshop held a few years ago to explore the possibility of "forecasting by analogy" — using the effects of recent natural and man-made changes as a guide to those that will occur in the future. It contains 15 case studies of varying relevance: they range from the rise and fall of the Californian sardine empire, already familiar to readers of John Steinbeck, through the fluctuating

fortunes of the lobster fishery in Maine, to the 'cod wars' between Britain and Iceland in the 1960s and 1970s. The problem with forecasting by analogy is that its utility depends on the aptness of the analogy, and the relevance of some of these cases, such as the last-mentioned, lies somewhere between arguable and dubious.

Nevertheless, there is much here of general historical interest, even if there are few solutions on offer, and the assiduous reader may find among the references, particularly in the introductory chapter by the editor, entry points to reasonably up-to-date literature on the subject. One surprising omission is any account of an attempt to apply empirical but quantitative biogeographical techniques to the problem. Given the difficulty of understanding the mechanisms at work, this may offer the best hope of practical predictions in the foreseeable future — once, that is, meteorological and oceanographic researchers can provide reasonably reliable predictions of sea-surface temperature and wind strength and direction on a regional scale.

In a final summary chapter, Michael Glantz and Lucy Feingold attempt to summarize the lessons to be learned from the case studies. Disappointingly, most of these are common sense, or restate the sanctity of motherhood and the palatability of apple pie. In the end, one finishes the book not much further forward than one started. I fear the truth is that there is little to offer policy-makers beyond the Boy Scout's advice "Be Prepared", and the scientific problems are such that this will be slow to change. □

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To boldly go

Grenville Turner

Lunar Sourcebook: A User's Guide to the Moon. Edited by Grant H. Heiken, David T. Vaniman and Bevan M. French. Cambridge University Press: 1991. Pp. 736. £50.00, \$59.50 (hbk).

REVIEWING a dictionary or an encyclopaedia is a difficult task, and so too with this book, which sets out to provide "a single, complete, and annotated description of our present knowledge of the Moon". If this book were a dictionary, however, one would have to point out that an important letter of the lunar alphabet, 'I' for isotope, is missing, but more of that anon.

The book is written by 25 contributors who have been immersed in lunar science for almost as many years. Three of the authors have acted as editors. The contents are a mixture of factual data summarized and systematized in tables, figures and text with relatively uncontroversial interpretation. The book is aimed at anyone, from scientist to space engineer, who wants to find out the current limits of our knowledge of the Moon. A short first chapter provides the reader with an introduction and a guide to using the book. The history of modern lunar exploration, culminating in the Apollo missions, is covered in the second chapter, which also introduces some of the main concepts of lunar science, from the giant impact theory of the Moon's origin to the early magma ocean and lunar differentiation. The reader is also introduced to the main lunar rock types and to the arcane details of lunar sample curation.

Chapter 3, ostensibly concerned with the external environment of the Moon, its transient atmosphere, meteorite bombardment and ionizing radiation, manages somewhat strangely to combine these topics with lunar heat flow, seismology and some aspects of lunar geology. For the geologist the meat of the book is in chapters 4 to 8, which deal with surface geological processes (impacts, volcanism and tectonics), lunar minerals, lunar rocks, the regolith and lunar chemistry, respectively. These are substantive and scholarly reviews and will form an ideal starting place for researchers or postgraduate students. Physical properties of the lunar regolith, particle sizes, density, compressibility and electromagnetic properties are covered in exhausting detail in chapter 9. Chapter 10 discusses geophysical and chemical data from orbital measurements, linking it to the regional geology and the geology of the Apollo landing sites. Twenty years after the last Apollo

landing, the concluding chapter makes a brave attempt to look forward to the future of lunar science, with a list of unanswered questions, future goals and schemes for permanent lunar bases.

The coverage of most areas of lunar science is so detailed and complete that it is really astonishing to have to comment on a really major omission, the absence of a serious attempt to discuss the vast array of isotopic data obtained from lunar samples. In the many and varied tables of data in the book, I found only one listing isotopic data. Also, apart from three or four figures summarizing schematically the conclusions of measurements on cores from the lunar regolith, I could not find any figures containing such data. The deficiencies are especially apparent in the superficial treatment of lunar age determinations throughout the book. Summaries of age measurements are largely confined to the chapter on lunar rocks, where 'ages' of highland rocks are tabulated according to breccia type. Because the petrological features relate mainly to local details of the impact, it makes little sense to discuss global lunar chronology in this context. Furthermore, the ages tabulated in chapter 6 do not even seem to be representative, there being no ages listed older than 3.95×10^9 years. Sm-Nd dating and the techniques of 'neutron stratigraphy', from which it was developed, are barely mentioned. Most serious of all, the fact that radiometric clocks carry important petrogenetic information on the relationship between chemistry and 'initial isotopic ratios' is completely overlooked. The reason for these and other omissions is clear from the list of authors, which does not include an easily identifiable expert in the field of isotope geochemistry.

For me the treatment of isotopes is a large disappointment, accentuated by the fact that in most other areas the book is first-rate. Writing the book clearly required a considerable effort by the authors and editors and it may be too much to hope that some future edition will remedy its one glaring deficiency. In spite of this, it is probably the best 'lunar dictionary' so far and an essential purchase for anyone with a serious interest in the Moon. □

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■ Elsevier have just published *Evolution of the Earth and Other Planetary Bodies* edited by R. Teisseyre, J. Leliwa-Kopystynski and B. Lang. This fifth volume in the series 'Physics and Evolution of the Earth's Interior' aims to provide cosmological insight into the formation and early evolution of the planetary system and the Earth. Dfl.370, \$211.50.

The calling of chaos

Robert M. May

Chance and Chaos. By David Ruelle. Princeton University Press: 1992. Pp.195. \$24.95, £19.95.

Searching for Certainty: What Science Can Know About the Future. By John L. Casti. Morrow/Scribners: 1992. Pp.496. \$12 (pbk), £16.99 (hbk).

WITH chaos and fractals featuring in a recent episode of the television detective series "Inspector Morse", it is clear that these topics are attracting wider attention. In London, one can visit a shop called Strange Attractions, which not only sells books, posters and T-shirts but also has a "chaos clinic" on Saturday mornings. (*The Sunday Times* said that the shop "will seduce a whole bizarre cross-section of people", while the *Evening Standard* said "it's weird but it's going to be big, very big".) The books reviewed here are two new entries in the chaos stakes.

David Ruelle, of the Institut des Hautes Etudes Scientifiques, just outside Paris, is one of the founders of the discipline. His book is a good translation of one that originally sold well in French. It is essentially a collection of some 26 short, connected essays (totaling 166 pages), along with a further 29 pages of much more detailed notes and comments set in smaller type. The book is aimed at the educated general reader and is largely free from jargon and equations (although some of the later chapters — for example those on quantum mechanics, equilibrium statistical mechanics and algorithmic complexity — do contain equations and, not only for this reason, are much more difficult than most of the earlier chapters).

Chance is the book's unifying theme. Beginning with conventional ideas about chance and probability, Ruelle leads us through the "deterministic randomness" of chaos (and its implications in physics, biology and economics), the assumptions about averaging that underlie statistical mechanics, the indeterminacy of quantum mechanics, and thence on to such things as Gödel's theorem of paradoxical undecidability in mathematical contexts and the role of unpredictability in the evolution of sex.

The book is an excellent read, either at one gulp or as chapter-by-chapter snacks. All the ideas are presented crisply and clearly, and even if one is thoroughly familiar with the material, one will enjoy the shameless digressions and the sardonic humour. One example (from the closing chapter): "what is the

origin of the urge, the fascination that drives physicists, mathematicians, and presumably other scientists as well? Psychoanalysis suggests that it is sexual curiosity . . . This explanation is somewhat irritating, and therefore probably basically correct. Sexual curiosity is at the root of science, but it is relayed by something else, namely the fact that the world is understandable." You may or may not agree, but this is one of umpteen asides that will keep coffee-time discussions lively.

John Casti is a member of the Institute of Econometrics, Operations Research and System Theory at the Technische Universität in Vienna. His book is considerably longer than Ruelle's (408 pages of main text, and 64 pages of notes and guidance for further reading) and, with its many graphs and other technical details, would seem to be aimed more narrowly, at a general audience of scientists. It is divided into six main sections, with the overall structure broadly paralleling that of Ruelle's book. In the first chapter, Casti treats "correlations, causes, and chance", discussing prediction and explanation in science and more generally. He begins with probability theory and statistics, and ends with chaos and strange attractors. The next four chapters give detailed accounts of four particular topics, with emphasis on chaos or on earlier work on catastrophe theory: climate and weather prediction; pattern formation and development ("from blobs to babies"); stock-market predictions (a review of older methods along with speculation about new ones based on strange attractors); and the theory of conflicts and wars ("wargasms as chaostrophes"). In the sixth chapter, Casti takes up philosophical questions about the nature of proof in mathematics, touching on Penrose tiling, Turing machines and Gödel undecidability on the way. He concludes the main text with brief thoughts about "what can we know for sure?"

In contrast with Ruelle's short and graceful essays, Casti's more detailed book is replete with all manner of typographic business. There are paragraphs with 'bullets', indented paragraphs set in smaller type, slogans in 'boxes', text presented as a dialogue between voices, along with tables, graphs, equations and cartoons. Each chapter ends with a summary, presented as a formal "term grade" for our ability in the various fields to predict and explain. The result is that the reader will find the average page visually lively, or cluttered, according to taste.

Both these books are excellent. Ruelle's repays reading, no matter how much or how little one already knows about chance and chaos. Casti's is good for those who want details, and is particular-

ly timely in providing a wide-ranging introduction to the possible, though debatable, relevance of nonlinear dynamics and chaos to predicting exchange rates, marginal rates of bonds and other economic indicators. □

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Physics in biology

Thomas A. McMahon

Newton Rules Biology: A Physical Approach to Biological Problems. By Colin Pennycuik. *Oxford University Press: 1992. Pp. 111. £20, \$49.95 (hbk), £9.95, \$19.95 (pbk).*

PHYSICS and biology were once the interests of the same scientists. Galileo, Descartes, Borelli, Boyle, Hooke, Poiseuille and Helmholtz all contributed to both. In the preface to this book, the author introduces A. V. Hill, a hero of modern-day animal physiologists, who began his career as a mathematician. Hill went on, in the early and middle part of this century, to become famous for showing how Newton rules biology; that is, how simple physical principles can be invoked to understand the results of experiments concerning muscle mechanics and energetics and the physiology of locomotion. Pennycuik says that one of his objectives in writing this book was "to show how Hill's way of thinking created a thread which links biological events at the cellular level, through animal locomotion, to the large-scale properties of ecosystems".

That is a big task, and he has made a substantial start in this slim volume. His subtheme within the physics-in-biology theme is the subject of physical dimensions. He begins by saying exactly what dimensions are, and how they are used and misused. Taking a number of examples from an important and delightful paper by Hill (the written version of a talk given as an evening discourse at the Royal Institution in 1949), the author discusses how the wing-beat frequency of birds varies with body size; why it would be expected that a two-metre fence would be sufficient for keeping both large and small antelopes in a confined space; and why fleas would need special rubber-like springs, as well as muscles, to jump. Along the way he finds room for developing insights about the mechanics of muscle. I particularly liked his elucidation of how one might calculate the energetic cost of maintaining tension

in one's own biceps muscle by finding experimentally the maximum shortening speed, dividing by the extended length, and dividing again by 16 to get the rate of energy consumption per cubic metre of muscle per pascal of stress maintained. I also liked the discussion of how the speed of muscles must be matched to the load. Human fingers, he says, have fast flexor muscles for rapid manipulation, and, as a result, people tire quickly when they hang from a window ledge. Orangutans, by contrast, can hang from their fingers for long periods but "are not suited to tasks that involve rapid finger movements".

Later, Pennycuik develops his dimensional theme with an introductory discussion of Mandelbrot's fractal geometry and what it might mean to biologists. In an example concerning the spacing of bald eagle nests on Amchitka and Adak Islands of the Aleutian Islands, he concludes: "the nest density is 2.6 times greater on Amchitka than on Adak, even when account is taken of Amchitka's more rugged coastline". This conclusion is available in spite of the curious fact that the spacing between nests is not, according to the author's arguments, "something which can be measured with a tape measure". Pennycuik ends with two chapters on dimensional aspects of ecosystems, including those that are modified by human activities. □

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New editions

- *Russian-English Translator's Dictionary: A Guide to Scientific and Technical Usage* by M. Zimmerman and C. Vedeneva, 3rd edn. Wiley, £65, \$139. For a review, see *Nature* **312**, 670 (1984).
- *Supermanifolds* by B. DeWitt, 2nd edn. For a review, see *Nature* **313**, 329 (1985). Cambridge University Press, £50, \$95 (hbk), £19.95, \$37.95 (pbk).
- *Physics of Massive Neutrinos* by F. Boehm and P. Vogel, 2nd edn. Cambridge University Press, £40, \$69.95 (hbk), £15.95, \$27.95 (pbk).
- *An Introduction to the Rock-Forming Minerals* by W. A. Deer, R. A. Howie and J. Zussman, 2nd edn. Longman, £21.99 (pbk).
- *Mountain Weather Climate* by R. G. Barry, 2nd edn. Routledge, £60 (hbk), £18.99 (pbk).
- *Plants and Microclimate: A Quantitative Approach to Environmental Plant Physiology* by H. G. Jones, 2nd edn. Cambridge University Press, £55, \$100 (hbk), £19.95, \$39.95 (pbk).
- *The Biochemistry of the Nucleic Acids* by R. P. Adams, J. T. Knowler and D. P. Leader, 11th edn. Chapman and Hall, £24.95 (pbk). For a review of the tenth edition, see *Nature* **326**, 223 (1987).

Water in the Earth's upper mantle

Alan Bruce Thompson

Experiments on the stability of hydrous minerals likely to be present in the Earth's mantle provide constraints on the distribution of water in the mantle, and the form in which it is stored. In regions of elevated mantle temperature, water may be stored not in minerals but in melts: such hydrous melts are important metasomatizing agents, and can induce volcanism beneath thick cratonic lithosphere.

WATER is present in all magmas and mantle rocks in the Earth today, albeit sometimes at the parts per million (p.p.m.) level. But it is not clear how this water is distributed within the mantle. There is also debate about whether water migrates as a free-fluid phase or dissolved in silicate melts.

The presence of water considerably lowers the melting temperatures of rocks. It also decreases magma viscosity and density, enhancing magma migration¹. Water in magmas greatly extends the compositional ranges of igneous rocks. The extent of these effects on the physical and chemical properties of magmas depends directly on the amount of water available.

Various estimates exist for the average water content of the mantle. Multistage heterogeneous accretion models for the Solar System yield values ranging from 1,000 p.p.m. (0.1 wt%; ref. 2) to 40 p.p.m. (ref. 3). Rubey's⁴ consideration of 'excess' volatiles (those too abundant in the atmosphere to be accounted for by rock weathering, and therefore derived from interior sources) led to an estimated average water content for the mantle of 400 p.p.m. Ringwood's⁵ modification for continental area suggests three times this amount. All considerations indicate that at least 90% of the Earth's water resides in the oceans and crust⁶. The remainder is presumably distributed heterogeneously in the core and mantle.

Magmatic water could originate from four sources: shallow recycled water from the hydrosphere, water assimilated from crustal rocks, water transported into the mantle by hydrous minerals in subducted oceanic lithosphere or stored primordial water. Many magmas assimilate shallow recycled water from the hydrosphere or from crustal rocks. This water can usually be distinguished from 'primary' or 'juvenile' mantle water by its stable isotope ratios⁷.

According to some estimates⁸⁻¹¹, subduction zones deliver six times more water into the mantle (8.7×10^{11} kg yr⁻¹, ref. 9) than is delivered to the surface by arc volcanism (1.4×10^{11} kg yr⁻¹, ref. 9). Although these figures cannot account for water stored in hydrous minerals in the mantle wedge, they imply that tremendous quantities of water bound in subducted hydrous minerals may be transported deeper than 100–125 km, the typically quoted depth of origin for arc magmas (see, for example, ref. 8). Here I address the question of how this subducted water is likely to be stored: as free fluid, in hydrous minerals, or in silicate melts at various depths in the upper mantle (35–410 km) and transition zone (410–660 km).

I suggest here that distinct water storage sites can be correlated with particular regions of the present mantle. This correlation is achieved by considering likely temperature distributions in different parts of the convecting mantle, relative to mineral stability and melting reactions. The following regions can be identified:

(1) In young (hot) subduction zones, water released by dehydration of the common hydrous minerals of oceanic lithosphere (mica, chlorite, amphibole, talc) is channelled back into the lithosphere through arc magmatism.

(2) In mature (cold) subduction zones, less-common hydrous minerals (such as phengite, pumpellyite, staurolite¹²) and the dense hydrous magnesium silicates (DHMS, Table 1) can be

important transporters of water at least down to the 660-km seismic discontinuity. Successive dehydration of DHMS in the slab at distinct depths could trigger melting in the overlying mantle wedge, and potentially also in the mantle transition zone.

(3) Alternatively, this dehydration water could become stored in other hydrous minerals that are stable in the regions of the mantle through which it passes, depending on the relative amounts of water and trace cations such as potassium. For example in potassium-rich regions of the transition zone, subducted water could become stored in K-amphibole. Depending on the stability of K-amphibole relative to the average current-mantle adiabat (ACMA) and on the amount of potassium in the transition zone, K-amphibole could be storing primordial water.

(4) Subcratonic lithospheric mantle xenoliths are richer than abyssal peridotites in potassium and other large-ion lithophile elements¹³. This enrichment is accomplished through metasomatism by a hydrous silicate melt. Such melts have depleted the upper mantle since the early Archean¹⁴. A mechanism for this enrichment probably involves thermal plumes.

(5) Rising plumes traversing the transition zone would first cause melting of K-amphibole-rich regions at ~410 km. This would be followed by dehydration of the nominally anhydrous

TABLE 1 Characteristics of dense hydrous magnesium silicates and other minerals mentioned

Phase	Abbreviation	Composition	Densities (g cm ⁻³)	Source (Ref.)
Hydrous-A	Hy-A	Mg ^{IV} Si ₂ O ₆ (OH) ₆	2.959	52, 81
Hydrous-B	Hy-B	Mg ^{VI} ₃ Si ^{IV} ₂ Si ₄ O ₃₈ (OH) ₄	3.368	52, 81, 82
Superhydrous-B	Su-B	Mg ^{VI} ₃ Si ^{IV} ₂ Si ₄ O ₁₄ (OH) ₄	3.21	81
Anhydrous-B	Anh-B	Mg ^{VI} ₃ Si ^{IV} ₂ Si ₄ O ₄₈	3.435	81
Hydrous-D	Hy-D	MgSiO ₄ H ₂ (?)	?	41
Hydrous-E	Hy-E	Mg _{2.27} Si _{1.20} H _{2.4} O ₆	2.89–3.2	42, 83
Hydrous-F	Hy-F	Mg _{1.2} Si _{1.8} H _{2.4} O ₆	?	42
Norbergite	nor	Mg ^{IV} Si ₄ O ₄ (OH) ₂	3.09	48, 81
Chondrodite	cho	Mg ^{IV} Si ₂ O ₆ (OH) ₂	3.06	81, 52
Humite	hum	Mg ^{IV} Si ₃ O ₁₂ (OH) ₂	3.15	48, 81
Clinohumite	chm	Mg ^{IV} Si ₄ O ₁₆ (OH) ₂	3.139	81, 52
10 Å-phase	10 Å	Mg ₃ Si ₄ O ₁₄ H ₆	2.65	41, 48
Serpentine	ser	Mg ₃ Si ₂ O ₅ (OH) ₄	2.55	84
Talc	tc	Mg ₃ Si ₄ O ₁₀ (OH) ₂	2.784	84
Anthophyllite	ant	Mg ₇ Si ₈ O ₂₂ (OH) ₂	2.953	85
Brucite	bru	Mg(OH) ₂	2.368	84
Forsterite	for (α)	α-Mg ₂ SiO ₄	3.213	84
Wadsleyite	β	β-Mg ₂ SiO ₄	3.69	15
Ringwoodite	γ	γ-Mg ₂ SiO ₄	3.72	15, 86
ortho-Enstatite	oen	MgSiO ₃	3.204	15
clino-Enstatite	cen	MgSiO ₃	3.19	84
Perovskite	pv	MgSiO ₃	4.15	15, 86
Stishovite	sti	SiO ₂	4.29	15, 86
Phlogopite	phl	KMg ₃ AlSi ₃ O ₁₀ (OH) ₂	2.784	81
K-richterite	K-ric	K _{1.9} Ca _{1.1} Mg _{0.5} Si _{7.9} Al _{0.1} O ₂₂ (OH) ₂	?	60, 61
K-amphibole	K-amp	K _{3.2} Mg _{6.5} Al _{0.5} Si _{7.0} O ₂₂ (OH) ₂	?	63
New K-silicate		(K _{1.1} Si _{2.7} O ₆ + 1–3 wt% H ₂ O)	?	63
Magnesiowüstite	mw	(Fe, Mg)O	(3.58)*	15
Clinopyroxene	cpx	Ca(Fe ²⁺ , Mg)Si ₂ O ₆	(3.32)*	86
Plagioclase	pl	(Ca, Na)(Al, Si)AlSi ₃ O ₈	(2.76)*	84
Chlorite	chl	(Mg, Fe) ₅ Al(Si ₃ O ₁₀ (OH) ₈	(2.8)*	85
Olivine	oi	(Mg, Fe) ₂ SiO ₄	(3.37)*	86
Orthopyroxene	opx	(Mg, Fe)SiO ₃	(3.3)*	86

* Densities for minerals with common mantle composition.

mineral β - Mg_2SiO_4 . This dehydration would generate water-undersaturated melt rather than appear as a free water fluid. Such melts will ascend to the lithosphere (even along the ACMA) and repeatedly enrich the lithosphere in large-ion lithophile elements.

To develop the arguments used here to detail the behaviour of water in the upper mantle and transition zone, it is first necessary to consider constraints on the thermal structure of the present-day mantle.

Temperature distribution in the mantle

The thermal structure of the mantle down to the base of the transition zone is constrained by combining results from high-pressure experimental petrology with depth, width and bulk modulus information for mantle seismic discontinuities (see, for example, refs 15 and 16). Figure 1 shows the ACMA which represents the horizontally averaged temperature profile in the slowly moving bulk of the upper mantle and transition zone. The surface potential temperature for the ACMA is $\sim 1,280^\circ\text{C}$, which is appropriate for the generation of mid-ocean-ridge basalt by decompression-melting of the mantle in response to extensional thinning of the oceanic lithosphere¹⁷. The ACMA is constructed by assuming that the 410-km seismic discontinuity is represented mainly by the α - β , γ transitions in $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ ^{5,15,18,19} at $\sim 1,450^\circ\text{C}$, and that the 660-km discontinuity is due largely to the transition of γ to $(\text{Mg}, \text{Fe})\text{SiO}_3$ -perovskite and $(\text{Fe}, \text{Mg})\text{O}$ near $1,600^\circ\text{C}$ ^{15,16,20}. The conduction thermal gradient for the lithosphere is omitted from the figures (but see Fig. 1 of ref. 17).

Mantle plumes need to be 100 – 400°C hotter than the ACMA to permit buoyant upwellings^{21–24}. A plume adiabat which is 200°C hotter than the ACMA is shown as PA in Fig. 1, with inflections appropriate to the exothermic 660-km and endothermic 410-km seismic discontinuities^{15,16}. No such inflections are shown for the ACMA because the bulk of the mantle is moving too slowly to influence the temperature distribution. Both the

ACMA and the PA are shown as dashed lines into the lower mantle although it is acknowledged that the 660-km discontinuity may coincide with a thermal boundary layer separating two differently convecting parts of the mantle^{15,25}. Although some numerical models^{21–23} suggest that the ACMA representing the geotherm through the central regions of mantle convection cells could be up to 200°C hotter than shown here, most observations on mantle melting support the indicated temperatures for the average and plume adiabats.

The base of the continental crust has an average depth of 35 km (Moho in Fig. 1). The ACMA meets the anhydrous (dry) peridotite solidus (DPS from refs 26–30 in Fig. 1) close to this depth. This suggests that melting of subcontinental mantle is regulated by crustal thickness^{17,31}. The average lithosphere thickness is ~ 125 km^{32,33}. The plume adiabat shown in Fig. 1, which has a surface potential temperature of $\sim 1480^\circ\text{C}$, meets the DPS at ~ 100 km¹⁷. Thus, some thinning of the lithosphere is necessary for the plume adiabat to cause melting at the DPS. Alternatively, a hotter plume adiabat (250°C) hotter than ACMA would be more appropriate. The present mantle thermal structure represented by the ACMA and the PA may not have changed much since the mid-Archean³⁴.

This simplified scenario suggests that the anhydrous peridotite solidus and the indicated adiabats are appropriate for melting of large regions of the mantle. But the presence of small amounts of water in most magmatic rocks^{8,35} and their enormous compositional diversity both indicate that volatiles in the mantle are fundamental to magmatism, and also imply that the volatiles are not evenly distributed in the mantle.

Release of volatiles by dehydration of subducting oceanic lithosphere has long been considered to be a key factor in arc magmatism (see, for example, ref. 8). Arc magmatism is related to subduction of oceanic lithosphere, but the details of the processes involved are still poorly understood (see refs 9, 36, 37). A large range of geotherms for the upper 15–20 km of the subducted slab (from ref. 38) is shown in Fig. 1. Most subduction

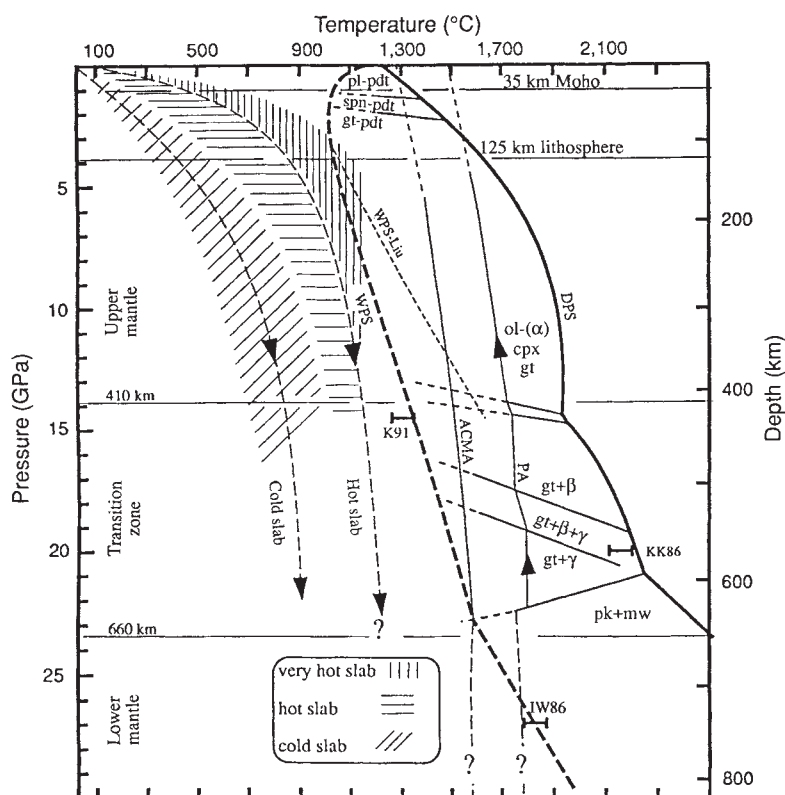


FIG. 1 Near-solidus petrology and temperature distribution in the upper mantle. The average current-mantle adiabat (ACMA) is constrained at the 410-km and 660-km seismic discontinuities near $1,450^\circ\text{C}$ and $1,600^\circ\text{C}$, respectively^{15,16}, and has a potential temperature of $1,280^\circ\text{C}$. The ACMA meets the anhydrous (dry) peridotite solidus (DPS)^{27–30} at a depth of 35 km¹⁷, in the plagioclase-peridotite field. An upwelling mantle plume adiabat (PA) which is 200°C hotter than the ACMA cuts the DPS near 100 km in the garnet peridotite field. The conduction gradients for the lithosphere are not shown (but see ref. 17). A new water-saturated (wet) peridotite solidus (WPS), constrained by the results from refs 42 and 43, lies at lower temperatures than one proposed by Liu⁴¹ (WPS-Liu). The new WPS shows inflections at the solid-solid phase boundaries and is roughly parallel to the DPS to a depth of 800 km. The three envelopes of subducting slab geotherms are obtained from ref. 38, Fig. 2. Mineral abbreviations are given in Table 1.

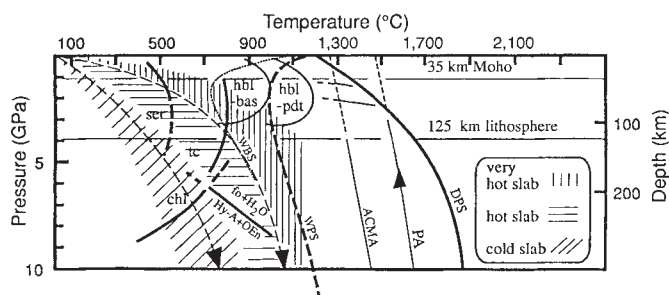


FIG. 2 Temperature-pressure diagram showing a simplified version of dehydration and melting for the three envelopes of slab geotherms. Dehydration of each of the slab components (metasediment, basaltic crust and peridotitic mantle) is considered to begin at ser (serpentine upper stability limit) and finish at tc (talc upper stability limit) with the important exceptions of hornblende in basalt (hbl-bas) or peridotite (hbl-per). Very-hot-slab geotherms result in melting of oceanic crust (at WBS) and perhaps of wet mantle (at WPS) shallower than 125 km. Hot-slab geotherms show a gap between tc and the formation of the dense hydrous magnesium silicates DHMS (Hy-A with oen). Water coexisting with fo will ultimately lead to wedge melting from about 125–250 km. Cold-slab geotherms result in transfer of water from common hydrous minerals (ser, chl, tc and so on) into the DHMS, and transport water deep into the transition zone.

geotherms are much colder than the ACMA at least down to the 660-km seismic discontinuity. If slabs pond at this depth then they will be heated to the ACMA. I will examine some recent results from high-pressure experimental petrology to establish where in the mantle water could be present as a free phase, where it is most likely to be stored in hydrous minerals and where it could be present in a melt.

A water-saturated mantle solidus to 800 km

Many experiments have shown that water considerably lowers the melting temperatures of rocks under pressure. It is important to establish at what temperature the mantle would melt if it were water-saturated. The early experiment^{26,37,38} to 6 GPa showed spectacular lowering of the solidus temperature of lherzolite (a rock type containing ol+cpx+opx+pl/spn/gt, considered representative of the upper mantle). For example, at 5 GPa the DPS at 1,800 °C was lowered to ~1,100 °C in the presence of excess water (the wet peridotite solidus WPS, in Fig. 1). Liu⁴¹ extrapolated these results to 15 GPa to provide a wet mantle solidus (WPS-Liu in Fig. 1). Two more-recent results^{42,43} have been used to extrapolate the water-saturated mantle solidus (WPS) through the transition zone.

From a mixture of $2\text{Mg}(\text{OH})_2 + \text{SiO}_2 = (\text{Mg}_2\text{SiO}_4 + 2\text{H}_2\text{O})$, Kanzaki⁴² produced melt at 1,300 °C and 14.5 GPa (K91 in Fig. 1). From their experiments on perovskite growth in the system $(\text{MgSiO}_3\text{--H}_2\text{O})$, Ito and Weider⁴³ proposed a solidus at 1,750 °C and 27 GPa (IW86 in Fig. 1). Although the water-saturated solidus (WPS) proposed in Fig. 1 is for the system $(\text{MgO--SiO}_2\text{--H}_2\text{O})$, it is some 500 °C lower than the DPS^{27–30} in the upper mantle and through the transition zone. The actual water-saturated mantle solidus will lie at even lower temperatures (perhaps 600 °C lower than the DPS) if the wet melting for Mg_2SiO_4 and MgSiO_3 is eutectic and if iron- and aluminium-bearing phases are considered.

Although it is based on very limited experimental data, the WPS is shown in Fig. 1 to be refracted (like the DPS) to higher temperatures at the solid-solid phase transitions. As the WPS and DPS are approximately parallel through the upper mantle and transition zone, it is possible to make a crude estimate of the water contents of water-saturated mantle melts. Because the extent to which the solidus temperature is lowered is proportional to the quantity of dissolved water in the melt, the solubility of water in melts through the transition zone should not be too different from that at the pressure region of the backbend in the WPS (~2–3 GPa). The experimental results from ref. 44 (for $\text{MgSiO}_3\text{--H}_2\text{O}$) and ref. 45 (for $\text{Mg}_2\text{SiO}_4\text{--H}_2\text{O}$) indicate ~20 wt% water solubility at melt-vapour saturation at 2 GPa. Thus, a water content of ~20 wt% seems likely along the entire WPS. If the mantle-water system is near-eutectic between Mg_2SiO_4 and MgSiO_3 ⁴⁶ (thus permitting maximum melt production), then melting at the WPS with, for example, 0.1 wt% water would generate ~0.5 wt% melt through the whole upper mantle and transition zone.

The ACMA meets the proposed WPS at ~660 km (Fig. 1). Any free water in the upper mantle and transition zone could thus be stored in small melt pockets, which would presumably

be more buoyant than surrounding mantle, and would therefore migrate upwards along the adiabat. Such melts should occur unless hydrous minerals are stable at temperatures higher than the ACMA, and thus store water in their structure.

Although the ACMA crosses the WPS at the depth of the 660-km seismic discontinuity, it has not yet been determined whether this has significance for the chemical evolution of the mantle through melting. Such evolution depends on the stability of the likely hydrous minerals relative to the WPS and to the thermal regime of the bulk mantle (ACMA). If no hydrous minerals are stable at temperatures higher than the WPS then 'subducted water', released by mineral dehydration in the slab, will migrate into the mantle wedge and induce melting at the WPS down to the 660-km discontinuity. Even if they are present only at ~0.5 wt%, such melts would migrate continuously through the upper mantle and impinge on the lithosphere, as long as they are less dense than their surroundings⁴⁷.

Finally, note that although at the top of the lower mantle the extrapolated ACMA (dashed in the figures) lies at lower temperatures than the proposed WPS, it cannot be certain that dehydration water released from slabs penetrating the 660-km discontinuity will exist as free water, because of a possible thermal boundary layer at this depth^{15,25}.

The aim now is to determine the stability of the likely hydrous minerals in the convecting mantle, and then to consider their abundance at various depths and thermal regimes. I will consider next the dehydration and melting behaviour of oceanic lithosphere in different kinds of subduction zones.

Shallow slab dehydration or subduction of volatiles

We would like to evaluate the conditions under which volatiles, stored in minerals, can be transported in subducted slabs past the depth of generation of arc magma (~125 km, refs 8, 9, 36–38). The problem is twofold. The first part concerns the pressure-temperature (*P-T*) stability of the common hydrous minerals in the various components of the oceanic lithosphere (meta-sediment, -basalt, -gabbro, -mantle). The second concerns the thermal structure in the hydrated part of subducted lithosphere, and in the overlying mantle wedge. This varies as a function of the age (temperature) of the lithosphere, the convergence rate, the maturity of the subduction zone and the rate of shear heating as primary parameters³⁸.

Far more is known about the dehydration boundaries in the least hydrated part of the lithosphere—the ultramafic mantle—than in the oceanic crust. Serpentine, talc and chlorite are the common hydrates in ultramafic compositions. Brucite ($\text{Mg}(\text{OH})_2$) is not present in mafic to ultramafic compositions at upper-mantle pressures⁴⁸. Dehydration boundaries for minerals in oceanic crust (amphibole, chlorite, epidote, chloritoid, pumpellyite and so on; see refs 12, 49) in mid-ocean-ridge basalt and metagabbro compositions, and phengite and biotite in subducted sedimentary rocks) are not well studied at upper-mantle pressures, despite a sedimentary component (¹⁰Be) in arc magmas⁵⁰. For present purposes the highest-temperature dehydration behaviour of the mafic and sedimentary components of the oceanic crust is modelled by the stability

limits for talc (tc) and chlorite (chl) in Fig. 2 (see ref. 36).

It is useful for the present discussion to distinguish three envelopes of slab geotherms ('very-hot', 'hot' and 'cold'). Temperature distributions for subduction of relatively young lithosphere (<20 Myr BP) ~10–20 Myr into the subduction history (path ∞ of ref. 38, Fig. 2b), lie close to the geotherm labelled 'Hot slab' in the figures. As a subduction zone matures, or when old lithosphere (>50 Myr) is subducted, geotherms approach the bound labelled 'Cold slab' (path D of ref. 38, Fig. 2a). The third envelope (very-hot) applies to subduction of very young (<10 Myr) oceanic lithosphere, recently produced at an ocean ridge, or with large amounts of shear heating (shear stresses of 60 to 100 MPa—path A of ref. 38, Fig. 2a). Note that previous discussions of fluid release during subduction (for example, refs 36, 41) have mainly considered only the very-hot-slab geotherms. Because of strong temperature gradients between the overlying mantle wedge and the relatively colder subducted lithosphere, and the fact that only the upper 20 km of the slab is likely to be hydrated (7 km oceanic crust, 13 km oceanic mantle), the illustrated geotherms apply to the upper few kilometres into the slab for various subduction histories (ref. 38).

Figures 1 and 2 show that only very-hot-slab geotherms cross the water-saturated solidus for metasediment and metabasalt (both represented by WBS in Fig. 2) and the solidus for the wet mantle (WPS in both figures). Dehydration of all slab components (sediments, oceanic crust and hydrated mantle) will occur at shallow depths (>3 GPa) for hot-slab geotherms. The water thus generated could cause water-saturated melting (see, for example, ref. 51) of eclogite (basaltic composition at >1 GPa) and could stabilize hornblende on the liquidus of eclogite and peridotite compositions at $P < 2.5$ GPa. The hornblende in eclogite (hbl-bas) will break down by dehydration-melting at depths of >90 km, contributing to arc magmatism (Fig. 2). It is to be expected that very-hot-slab geotherms result in the return of most subducted volatiles to the crust via arc magmatism.

Hot-slab geotherms (Fig. 2) do not cross the WBS. But dehydration of serpentine and talc in the hydrated mantle in hot slabs could induce melting both in eclogitic oceanic crust⁵¹ and in the overlying mantle. As can be seen in Fig. 2 (and in more detail in Fig. 3 of ref. 48), there is a region between 4 and 8 GPa for hot-slab geotherms where free water coexists with olivine. Thus in the depth range 125–250 km, dehydration water will not be retained in the 'hot slab': the released water could result in melting of eclogitic oceanic crust and possibly of the mantle wedge, depending on the detailed thermal structure.

Cold-slab geotherms, appropriate to subduction of >50 Myr oceanic lithosphere in mature (>50 Myr) subduction zones, are the best candidates for subduction of water deep into the mantle. It has been suggested that dense hydrous magnesium silicates

could provide water-storage sites in the mantle. Along cold-slab geotherms, dehydration of serpentine and talc produces the DHMS phases 10 Å and Hy-A (Table 1)⁴⁸, rather than olivine plus free water. Experiments to date on the simple system MgO–SiO₂–H₂O (see, for example, refs 48, 52) apply mainly to the hydrated oceanic mantle. To understand how much water can be subducted past the depth of arc magma generation requires new experimental work between 400 and 700 °C, at 4–10 GPa to determine the stoichiometry of the mineral reactions on compositions that reflect the three hydrated-slab components (sediment, oceanic crust and mantle). Only then can we attempt a realistic mass-balance of how much water is subducted into the deeper mantle in 'cold slabs', past the depth (~125 km) of generation of arc magmas.

DHMS and deep water storage

Since it was first reported that DHMS were found when the simple system MgO–SiO₂–H₂O was subjected to high pressure and temperature (refs 48, 53), there have been repeated suggestions (refs 41, 54, 55) that such minerals (Fig. 3) might be able to store water deep in the mantle. The reconnaissance study by Liu⁵⁵ of the breakdown products of serpentine using a laser-heated diamond anvil suggests that a range of DHMS assemblages could be stable to different depths in subducted oceanic lithosphere at least as far down as the 660-km seismic discontinuity (shaded region in Fig. 4).

The DHMS are identified by alphabetic abbreviations prefixed with Hy to emphasize their hydrous nature (see Table 1). Apart from differences in water-content, these silicates show a large range in silica content compared with a simplified mantle composition (between fo and oen with some water—the exaggerated shaded region in Fig. 3). Hy-A and Hy-B are, like the humite minerals (nor, cho, hum, chm in Table 1), very low in silica, whereas Hy-D, Hy-E, Hy-F and 10 Å are relatively rich in silica.

Most experimental studies on DHMS have been done between 500 and 1,200 °C (or occasionally 1,300 °C), and between 3 and 28 GPa; these physical conditions are appropriate for subducted slabs, but not for the rest of the mantle. Even in subducted slabs, water may be bound in the DHMS or present as a free phase, depending on the age (temperature) of the slab. I will concentrate for the moment on relatively hot-slab geotherms and distinguish the regions of the mantle down to 300 km from the range 300–600 km. We should remember that the water budget in 'hot slabs' is diminished relative to cold slabs, because of magmatism.

Water storage in the upper mantle. To designate 'water-storage lines' in the mantle, both Liu⁴¹ and Ahrens⁵⁴ have extrapolated the early multi-anvil experimental data from refs 48, 52 to higher pressures and temperatures. They determined the synthesis fields of the DHMS Hy-A and Hy-B and the humite mineral chondrodite (cho) relative to fo, oen, periclase (per) and water in

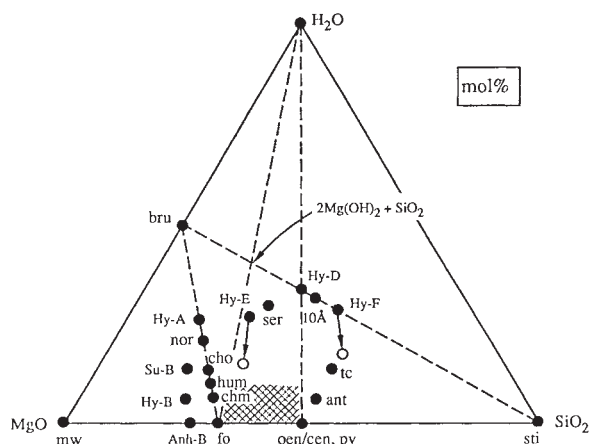
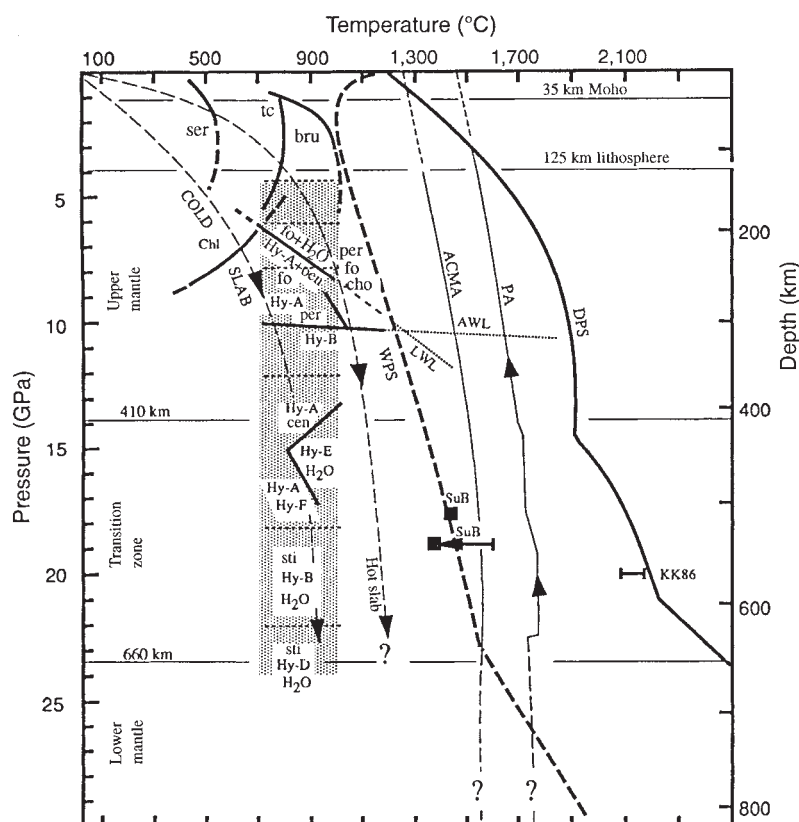


FIG. 3 Molar plot of the compositions of dense hydrous magnesium silicates (DHMS) relative to commoner minerals in the system MgO–SiO₂–H₂O (see abbreviations in Table 1). The arrows for Hy-E and Hy-F show recalculations for various estimated water contents. The shaded region shows the composition range of hydrous mantle with an exaggerated water content.

FIG. 4 Temperature–pressure diagram showing experiments on $\text{MgO-SiO}_2\text{-H}_2\text{O}$ phases relative to the DPS and WPS and mantle temperature distribution (Fig. 1). The shaded region corresponds to the reconnaissance work on serpentine breakdown⁵⁵ and the solid lines within this band shows the results from refs 48, 52 and 42. The ‘water lines’ AWL (Ahrens⁵⁴) and LWL (Liu⁴²) were obtained by extrapolation of the data of refs 48 and 52 to higher temperatures. Mineral abbreviations are given in Table 1.



the range 750–1,200 °C, 8.5–16 GPa. Liu's 'waterline' (LWL in Fig. 4) was obtained by extrapolating the data from ref. 52 (experiments between 750 and 1,200 °C), to ~1,500 °C and 12 GPa. This 'waterline' favours water storage in Hy-A with pyroxene deeper than 200–300 km at the expense of $\text{Mg}_2\text{SiO}_4\text{-H}_2\text{O}$, depending on temperature. Ahrens's⁵⁴ 'waterline' (AWL in Fig. 4), obtained by extrapolation of the data from ref. 48 to 1,900 °C and 12 GPa, favours Hy-B as the water storage site deeper than 300 km.

According to these models, water would be stored in the DHMS (Hy-A or Hy-B) deeper than 200–300 km but would be present as a free fluid phase coexisting with fo or oen at shallower depths. But both the Liu⁴² and Ahrens⁵⁴ 'water storage lines' (LWL and AWL in Fig. 4) meet the proposed WPS near 1,250 °C (10.5 GPa). This implies that any free water exists only at temperatures below the WPS and would easily become dissolved in mantle melts at higher temperatures in and near subducted slabs. Thus the 'water storage lines' near 300 km at best represent the region in the mantle where water is dissolved in melts rather than being stored in minerals.

The recent multi-anvil experiments by Kanzaki⁴² and Luth⁵⁶ have a bearing on this problem. Both these studies showed that Hy-A is not stable above 1,000 °C from 6 to 15 GPa, and those by Luth also showed that chondrodite (cho in Figs 3 and 4) is not stable above 950 °C at 9–10 GPa. These dehydration boundaries lie at noticeably lower temperatures than the WPS (Fig. 4). Thus water released by dehydration in subduction zones (hot to cold) will migrate into the overlying mantle wedge and could ultimately trigger melting in this region. I have discussed the role of slab dehydration in the generation of arc magmatism in the previous section. For the moment it is appropriate to follow the DHMS behaviour deeper into the mantle.

Water storage in DHMS in the mantle transition zone. The reconnaissance experiments of Liu⁵⁵ on serpentine up to 28 GPa at 700–1,000 °C indicate that a range of DHMS coexists with stishovite (sti) and brucite (bru) over different pressure ranges (Hy-A to 18 GPa, Hy-B from 18 to 22 GPa and Hy-D from 22 to 28 GPa. As noted above, the multi-anvil experiments by

Kanzaki⁴² have shown that Hy-A is not stable above 1,000 °C from 6 to 15 GPa. They also demonstrated that Hy-B may be stable to temperatures above the WPS between 10 and 15 GPa, but that it will not appear in the composition range of the mantle. It remains to be seen whether Hy-E and Hy-F are stable in mantle compositions above 1,000 °C. According to the data in ref. 55, Hy-D is stable in subducted slabs near the 660-km seismic discontinuity. The thermal stability of Hy-D needs to be experimentally investigated through multi-anvil work, as does the relation of phase Hy-E (ref. 42) to phase Hy-D (ref. 55).

Do other components stabilize DHMS in the mantle? No experiments so far have considered the effect that other important components (mainly iron, aluminium and calcium) may have in changing the stability of dehydration and melting of hydrous mantle into the transition zone. As noted above, these components would displace the present WPS (proposed only for experiments on $\text{MgO-SiO}_2\text{-H}_2\text{O}$) to lower temperatures, because iron partitions preferentially into mantle melts rather than residual minerals. Furthermore, depending on the partitioning behaviour of iron and magnesium among DHMS, some dehydration boundaries could be displaced to higher temperatures than in the simple system $\text{MgO-SiO}_2\text{-H}_2\text{O}$. These displacements are only significant for water behaviour in the mantle if common mantle-hydrates are stable to higher temperatures than the 'impure' WPS (the WPS that allows for the influence of iron, magnesium, calcium and so on). The highest-temperature stability reported for DHMS in the transition zone is for superphase B (Su-B, Table 1). Gasparik⁵⁷ reported Su-B at 17.8–18.2 GPa, 1,450 °C, but as no water was added to the sample he presumed that Su-B ($\text{Mg}_{10}\text{Si}_3\text{O}_{14}(\text{OH})_4$) was stabilized by fluorine from the PbO-PbF_2 flux used. Thus, the only current example of a DHMS being stable at temperatures higher than the proposed WPS in the transition zone is a fluorine-stabilized superphase-B.

Dehydration deep in subducting slabs. An upper limit of 1,000 °C for the thermal stability of the DHMS would mean that these minerals would be able to store water in deep subducted slabs but not in the rest of the mantle. A series of DHMS reactions

can be envisaged at distinct depths in subducted slabs, corresponding to the thermal structure of particular subduction zones. It has been proposed⁵⁸ that deep earthquakes are the result of dehydration reactions. For slabs ponding at the 660-km discontinuity the breakdown of Hy-D will govern the dehydration at the bottom of the mantle transition zone. Continuous subduction of ocean floor in mature subduction zones can feed a mineral reservoir of water into the transition zone of the mantle. This will result in a patchy water reservoir through time as subduction zones cease and start anew elsewhere.

If the DHMS are only stable in subducted slabs but not in the rest of the mantle, are there other stable hydrous minerals that could store water? There are two aspects to this question. First, are such hydrous minerals stable to temperatures higher than the WPS—in which case infiltrating water would first make new hydrous minerals which would then later melt at higher temperatures? Second, are any hydrous minerals stable to temperatures higher than the ACMA? If so, such minerals could store primordial water in those parts of the mantle that have never been traversed by rising plumes.

Other hydrous minerals in the mantle?

According to ref. 59, phlogopite is stable at least to 1,300 °C, 4 GPa in a peridotitic mantle (Fig. 5). The quantity of phlogopite depends on the amount of potassium and water relative to the stoichiometric K/OH ratio of mica. An excess of potassium would require the presence of additional potassium-bearing minerals; an excess of water could form other hydrous minerals or water-bearing melts.

It has recently been confirmed⁶⁰, following the suggestion advanced in ref. 61, that K-richterite is more stable than

phlogopite plus diopside (phl+dio) at pressures of >6 GPa. The experiments showed that phl+dio is replaced by K-richterite (K-ric in Fig. 5)) with ol, cpx, gt over a wide reaction interval from 6 to 11 GPa, 1,000 to 1,300 °C. Note that Sudo and Tatsumi⁶⁰ did not report melting, implying that K-richterite could be stable some 200 °C higher than the proposed WPS (Fig. 1) in mantle compositions. More recently Trønnes^{62,63} has shown that K-richterite is replaced by a low-aluminium, high-potassium amphibole (K-amp in Fig. 5) at ~13–14 GPa, coexisting with another hydrated K-silicate (Table 1).

A phlogopite-peridotite solidus (PPS) inferred from refs 59 and 64 has been extended to higher pressures into a K-amphibole dehydration-melting region (KPS) in Fig. 5, consistent with the experiments in ref. 65. New experiments on likely mantle compositions are needed in the ranges 1,100–1,600 °C and 10–20 GPa to locate the PPS and KPS correctly. Nevertheless we can use the illustrated solidi to consider the role of these K-bearing hydrous minerals in the mantle water storage problem. By chance, the KPS shown in Fig. 5 crosses the ACMA near 410 km. If the KPS is correctly located, then K-amphibole⁶³ could store water in the upper mantle and into the transition zone. Clearly the amount of K-amphibole depends simply on the amount of potassium. It has been proposed⁶⁶ that the amount of potassium in the transition zone is about 660 p.p.m., a value determined by assuming that atmospheric argon is degassed from the upper mantle down to 410 km. The amount of potassium in the mantle is further limited by implied radiogenic heat production^{67,68}, and 1,000 p.p.m. is consequently an upper bound³³. For the K-amphibole in Table 1 this would only correspond to ~200 p.p.m. water.

At depths of <410 km, as the ACMA lies at higher temperatures than the illustrated PPS and KPS in Fig. 5, water would be stored in melts. A plume adiabat would intersect an extension of the illustrated KPS near the 660-km discontinuity. Dehydration-melting of K-amphibole at the KPS by plumes traversing the transition zone is a potential source for mantle melts. The highly potassic and volatile-rich inclusions in diamonds from Zaire have been interpreted as mantle melts^{69,70}, quenched to mica, carbonate and apatite with excess H₂O + CO₂. It is possible that these are the metasomatic 'fluids' that generate mica-amphibole-rutile-ilmenite-diopside (MARID) and glimmerite xenoliths and lead ultimately to kimberlite eruption (see, for example, ref. 71).

But even if it is unevenly distributed, there is insufficient potassium in the mantle to stabilize large quantities of K-amphibole except locally, and it is known that clinopyroxene can contain 1.5 wt% K₂O (ref. 72) in the upper mantle. Even though the DHMS may not be stable in the bulk of the mantle, their compositions are such (Fig. 3) that they could be present in large quantities in mature subducted slabs. I turn now to the question of whether or not the common nominally anhydrous minerals in the upper mantle and transition zone can store water.

Water in nominally anhydrous minerals

As Bell and Rossman⁷³ recently stated, most of the nominally anhydrous minerals (NAMS) in the mantle contain structurally bound OH. Pyroxenes contain 200–500 p.p.m. water⁷⁴, and β -Mg₂SiO₄ has been found to contain up to 4,000 p.p.m. (0.4 wt%) water⁷⁵. Bell and Rossman⁷³ concluded that the water content of mid-ocean-ridge basalt (100–200 p.p.m. water) and of arc magmas (2 wt% water²⁵) can be derived entirely from melting of NAMS. The important issue to be resolved is how the melting and dehydration reactions of (OH)-bearing NAMS compare to those in totally anhydrous mantle. It might be argued (following ref. 76) that even experiments on anhydrous mineral powders always involve surface-adsorbed water. Alternatively, it could be suggested that the eutectic 'hydrous' melting of Mg₂SiO₄–MgSiO₃⁴⁶ at 2,150 °C, 20 GPa (KK86 in Fig. 1) reflects the melting of OH-bearing β -Mg₂SiO₄. Clearly the results of ref. 46 lie within the uncertainties of temperature calibration

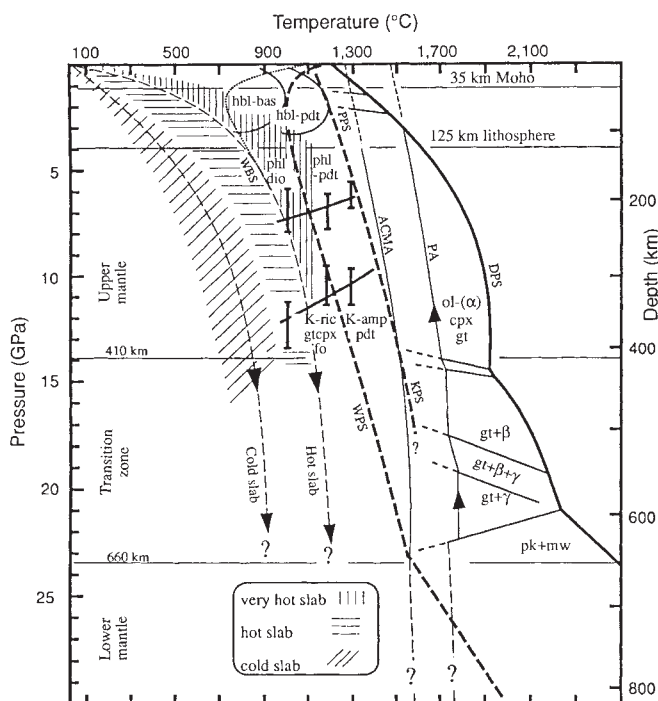


FIG. 5 Temperature–pressure diagram showing results on amphiboles (K-ric⁶⁰, K-amp^{62,63}) and phlogopite (phl⁵⁹) relative to the WPS, for the present mantle temperature distribution (Fig. 1). Although the phlogopite–peridotite solidus (PPS) is some 200 °C above the WPS, it lies at lower temperature than the ACMA in the upper mantle, so that phlogopite will only be stable in the lithosphere. The estimated K-amphibole peridotite solidus (KPS) cuts the ACMA near 410 km, indicating that such amphiboles may be stable in the transition zone. Mantle plumes (PA) would first cause melting of K-amphibole and then dehydration of β -Mg₂SiO₄. These small amounts of melt will aid plume ascent through the upper mantle.

compared to the DPS (refs 27–30). There is no reason at the moment to expect that the melting reactions of OH-bearing NAMS occur at temperatures much below the DPS.

In regions of the mantle transition zone that do not contain potassium, water is most likely to be stored as OH in β -Mg₂SiO₄. Because the thermal stability of OH-bearing β is probably not too different from truly anhydrous β , it will be stable up to temperatures of the DPS and certainly could be a storage site for primordial water in the transition zone. The storage site for water in the upper mantle is most likely to be cpx⁷⁴, rather than gt or ol⁷³.

A plume rising along the plume adiabat shown in the figures as PA (200 °C above ACMA) will induce dehydration (dehydration) of β at ~410 km. Because OH is more soluble in β -Mg₂SiO₄ than in cpx, gt or ol, the small amounts of OH released will cause small degrees of melting at the top of the transition zone. The degree of melting will aid plume ascent and will increase as the plume migrates upwards. This will occur mainly because a plume rising along the PA through the upper mantle progressively approaches the DPS, and because the PA will cross isosolubility lines for water in mantle melts. Such isosolubility lines run roughly parallel to and between the WPS and DPS, decreasing from about 20 wt% at the former (see above) to zero at the latter.

Conclusions and suggestions

The location of the water-saturated peridotite solidus (WPS), relative to the three major thermal regimes in the mantle (subducting slabs, bulk mantle and mantle plumes), permits us to envisage the likely ranges of water behaviour in the mantle down to the 660-km seismic discontinuity. Subduction of 'very-hot slabs' (new subduction zone with very young oceanic lithosphere) leads to dehydration of oceanic crust and hydrated mantle shallower than 125 km. This water is returned to the surface by arc volcanism or becomes stored in the lithosphere in and around intrusives.

'Hot slabs' (young subduction zones with still-hot oceanic lithosphere) encounter a gap between water storage in the common hydrous minerals of the subducting lithosphere (micas in metasediments; amphibole, chlorite and chloritoid in metabasalt; serpentine, talc in hydrated mantle) and the dense hydrous magnesium silicates (Hy-A, Hy-B). In this gap (600–1,100 °C, 3–10 GPa) free water is stable with olivine. This dehydration water escapes the slab and can induce melting in eclogitic oceanic crust and eventually in the overlying mantle wedge down to 300 km. New experiments are needed in the quoted pressure-temperature range to see how additional components influence the stability field of fo-H₂O. Iron, for example, should increase the stability of olivine at the expense of all other common hydrous minerals (except anthophyllite) occurring in hydrated mantle compositions, whereas potassium will stabilize phlogopite or K-amphibole at the expense of olivine-water.

'Cold slabs' (mature subduction zones with old oceanic lithosphere) could result in direct transfer of stored water from the common hydrous minerals of the oceanic lithosphere into the DHMS. New experiments are needed in the range 400–800 °C, 3–8 GPa to see how the common hydrous minerals of metasediments, metabasalt and hydrated mantle overlap with the DHMS. Only when reaction stoichiometries are known can a meaningful mass-balance for the likely amount of subducted water be attempted. A range of DHMS minerals are expected to dehydrate at distinctly different depths in mature slabs at least down to the 660-km seismic discontinuity. If deep earthquakes are due to these dehydrations, then correlations are expected with depth and with the thermal maturity of subduction zones. A knowledge of the partitioning of iron and magnesium among the DHMS is required to understand how the reaction boundaries in Fig. 4 become displaced with depth in subducted slabs.

The behaviour of water in the bulk of the mantle down to the 660-km seismic discontinuity away from subduction zones is

harder to assess, because of the lack of experimental data. Water released by dehydration of DHMS in mature slabs can be stored in K-amphibole and β -Mg₂SiO₄ at least in the depth range 410–500 km. The significance of K-amphibole in the transition zone is difficult to determine for several reasons. First, the location of the KPS in the transition zone is not known—the pressure-temperature range for future experiments is suggested from the provisional location in Fig. 5. Second, the expected amount of potassium in the transition zone would only make small amounts of K-amphibole. Third, K-amphibole may be an important mineral only near subducted slabs if the dehydration water also transports high quantities of potassium, as proposed for shallower depths in the mantle^{36,73,78,79}.

If slabs pond at 660 km and dehydration of Hy-D occurs (Fig. 4), then the fate of the resulting water is not easy to ascertain. The ACMA crosses the WPS close to this depth, the stability of K-amphibole is not known and β -Mg₂SiO₄ is not stable. The bottom of the transition zone may involve hydrous melting of megaliths⁸⁰. But a mass-balance for slab-water is needed at this depth, as are the OH-contents of perovskite and magnesio-wüstite relative to γ and β -Mg₂SiO₄.

The behaviour of water at the top of the transition zone (~410 km) is somewhat easier to envisage than at the bottom (~660 km). The ACMA crosses the proposed KPS near this depth, implying that subducted and primordial water could be stored in K-amphibole if there is enough potassium in the mantle, and in β -Mg₂SiO₄ if there is not. Plume ascent (PA) across the 400-km seismic discontinuity will first decompose K-amphibole then β -Mg₂SiO₄. Melts associated with plumes reach the lithosphere, especially if they have sampled hydrated mantle.

The long-term behaviour of the upper mantle (down to 410 km) seems to be one of recycling water, released by dehydration in subduction zones, through melts back to the lithosphere. Because the ACMA lies at higher temperature than the proposed KPS and PPS, K-amphibole and phlogopite may form transiently in the mantle wedge, but will eventually be melted as the wedge attains the temperatures of the ACMA. One final conclusion is that the major metasomatizing agent in the bulk of the mantle (away from subduction zones) seems to be hydrous melt rather than aqueous fluid. □

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ARTICLES

The folding of hen lysozyme involves partially structured intermediates and multiple pathways

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Analysis of the folding of hen lysozyme shows that the protein does not become organized in a single cooperative event but that different parts of the structure become stabilized with very different kinetics. In particular, in most molecules the α -helical domain folds faster than the β -sheet domain. Furthermore, different populations of molecules fold by kinetically distinct pathways. Thus, folding is not a simple sequential assembly process but involves parallel alternative pathways, some of which may involve substantial reorganization steps.

UNDERSTANDING how a globular protein folds requires detailed characterization of partially organized intermediates formed during the folding process. Information about such species is becoming available through studies of disulphide formation in oxidative refolding^{1,2}, protection from hydrogen exchange^{3–6}, the effects of amino-acid substitutions^{7–9}, stable partially folded states^{10–13} and peptide analogues of folding intermediates^{14,15}. Nonetheless, no clear unifying view of the nature of folding mechanisms has yet emerged and uncertainties remain over several fundamental issues.

Here we describe a detailed study of the refolding of hen lysozyme. The thermodynamics and kinetics of folding of this protein under equilibrium conditions accord well with a cooperative two-state model^{16,17}. Under conditions far from equilibrium, however, refolding occurs in at least two stages, acquisition of a native-like far ultraviolet (UV) circular dichroism (CD)

spectrum preceding formation of the tertiary structure, as monitored by near-UV CD^{18,19}. In addition, a very fast phase has been detected by tryptophan absorption measurements²⁰.

These results suggest transient population of partially folded intermediates but their detailed interpretation requires complementary information about the behaviour of individual residues, such as can be obtained from hydrogen-exchange labelling techniques^{5,6,21}. Using a variant of these experiments^{3,4}, based on competition between the exchange and folding processes, we have recently confirmed the existence of folding intermediates for hen lysozyme and demonstrated that the two structural lobes that characterize the native structure are also distinct folding domains²². Here we describe pulsed hydrogen-exchange labelling experiments, using nearly half of the 126 amide hydrogens in the molecule as probes of refolding, and stopped-flow CD measurements in both far- and near-UV

spectral regions. The combination of these data has allowed us to construct a detailed analysis of events during refolding.

Evidence for partially folded intermediates

Figure 1 shows the results of pulsed labelling experiments obtained for individual amides, grouped according to their distribution in the native state secondary structure. If folding occurred by a simple two-state mechanism all the curves would be coincident. The fact that this is not the case demonstrates unequivocally that partially structured intermediates are populated during folding.

The different labelling curves are not only distinct for the different sets of amides, they are also not monophasic. Each curve was modelled well by a sum of two exponentials (Fig. 1 and Table 1). The rates of the fast phases show no very clear pattern (time constant (τ) = 7 ± 4 ms) but those of the slower phases fall qualitatively into two groups, differing in their average time constant by a factor of about 4. The more rapidly protected group ($\tau < 100$ ms) comprises amides in the four α -helical segments, the 3^{10} helix close to the C terminus of the protein, and three amides, Trp 63, Cys 64 and Ile 78, which lie in the loop region in the native enzyme. With the exception of the last group, these structural elements all occur in one of the two lobes of the native conformation, which we shall call the α -domain (Fig. 2). In contrast, amides protected more slowly

($\tau > 100$ ms) are located, with the single exception of Asn 27, in the other structural domain, which we designate the β -domain, comprising a short double-stranded and a longer triple-stranded β sheet, a 3^{10} helix and a long loop.

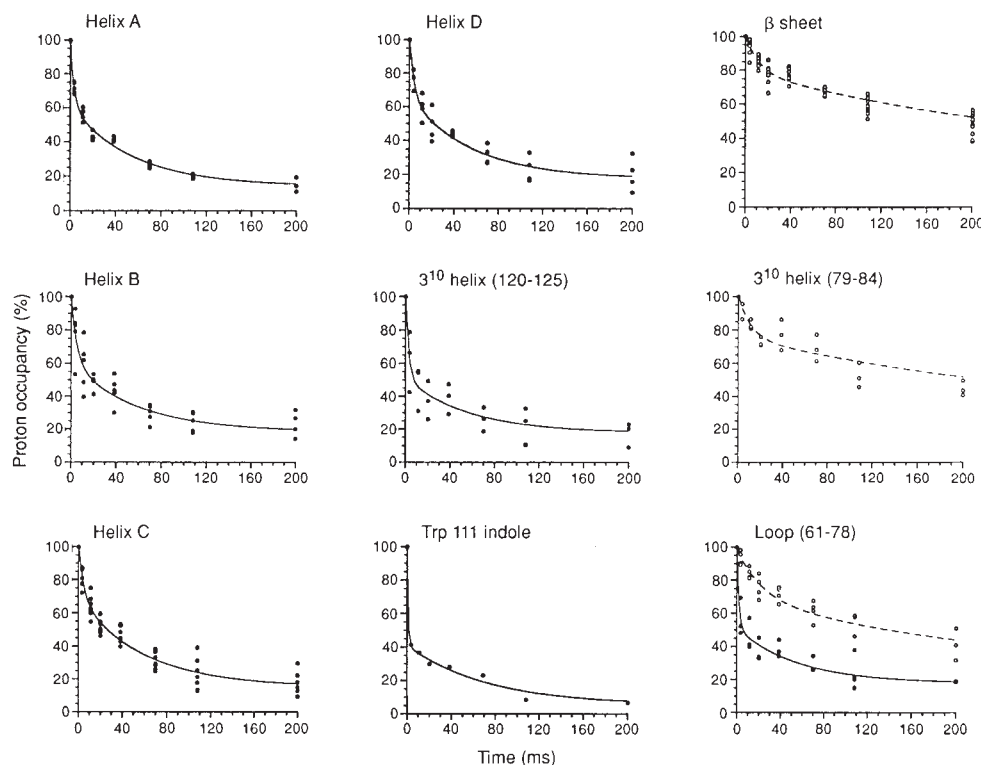
The same qualitative distinction also extends to the amplitudes of the kinetic phases. For amides in the α -domain, the fast phase constitutes some $40 \pm 12\%$ of the total whereas in the β -domain the corresponding amplitude is only $24 \pm 6\%$. This compounds the difference in kinetics of the second phase to ensure that protection of the β -domain is substantially slower overall, thus accounting for the pattern of labelling observed when the refolding of lysozyme was examined by the competition method²².

Tertiary interactions and folding domains

The similar protection curves for nearly all amides in the α -domain could, in principle, mean that individual helices form as persistent structures independently but simultaneously. However, the coincidence of both kinetic phases makes this seem unlikely. A more plausible explanation is that the protection from exchange monitors stabilization of the rapidly formed helices by side-chain interactions, and hence that these amide probes actually monitor formation of the domain core, albeit perhaps in an embryonic form. This is consistent with the protection of two amides not directly involved in secondary structure, Leu 17 and Tyr 23 which lie in the loop linking helices

FIG. 1 Time courses for the protection of amides from exchange during the refolding of hen lysozyme. The curve drawn represents the average of a two exponential fit to the time course for individual amides in each native secondary structural element. In the case of the loop region, two averages are drawn, one for Trp 63, Cys 64 and Ile 78 and another for the remainder.

METHODS. Experiments were done at 20 °C using a Biologic QFM5 rapid mixing quench flow apparatus. Lysozyme (20 mg ml⁻¹) was initially dissolved in 6 M guanidine deuteriochloride in D₂O at pH 6.0, leading to complete denaturation and substitution of all exchangeable hydrogens by deuterium. Refolding was initiated by dilution of this solution 10-fold into 20 mM sodium acetate, pH 5.5 in H₂O. At the resulting pH of 5.2 the half life for amide exchange is about 16 s (ref. 32) so that negligible labelling occurred during this phase. After variable refolding times (3.5–2,000 ms) the solution was diluted again with a volume 5 times that of the initial protein solution of 0.2 M sodium borate, pH 10.0. This initiated labelling at a pH of 9.5. After 8.4 ms the labelling pulse was terminated by further dilution, with a volume again 5 times that of the initial volume of protein solution, of 0.5 M acetic acid in H₂O. The final pH was about 4.0, at which exchange in the native protein of the 49 amides studied is very slow²⁶. A sixfold lower protein concentration during refolding had no effect on the observed rates of protection, confirming that intermolecular associations, if present, are not kinetically significant. Protein samples were concentrated and the buffer exchanged for 40 mM deuterated sodium acetate, pH 3.8 in D₂O by ultrafiltration at 4 °C. For the zero time point (100% labelling) a solution of lysozyme in the same buffer and isotopic composition as in the labelling phase of the refolding experiment was heated to 80 °C for 10 min, causing complete exchange of all amides by reversible unfolding. The sample was then treated identically as for the other time points. This made correction for the residual deuterium content of the labelling solution unnecessary. A phase-sensitive COSY spectrum of each sample was recorded



on a 500 MHz GE/Nicolet spectrometer at 35 °C. 256 increments over 1,024 complex points and 32 transients were collected. Data were processed using the FTMNMR program of Dennis Hare (Hare Research Inc.) on a SUN computer. Final digital resolution was 1.7 Hz per point. Acquisition and processing of each spectrum was identical. The intensities of the α H-NH cross-peaks in each spectrum were taken as the sum of the absolute values of the four phase-sensitive components. These were normalized using the sum of the eight phase-sensitive peaks associated with the Tyr 23 and Tyr 53 C δ H-C ϵ H cross-peaks. Proton occupancies at individual amide sites were calculated relative to those of the unfolded control sample, for which COSY cross-peak intensities were taken to correspond to 100% proton occupancy.

TABLE 1 Protection of amide hydrogens during the refolding of lysozyme

Residue	Fast phase		Slow phase		Structural context	Residue	Fast phase		Slow phase		Structural context
	Amplitude (%)	Time constant (ms)	Amplitude (%)	Time constant (ms)			Amplitude (%)	Time constant (ms)	Amplitude (%)	Time constant (ms)	
8	47.2	3.90	38.3	69.8	Helix A	61	17.5	9.29	58.5	247	Loop (irregular)
10	39.5	4.41	50.6	64.3		63	60.0	2.45	25.4	63.9	
11	43.6	3.39	41.5	54.1		64	43.2	3.67	37.4	60.4	
12	33.9	2.61	51.5	53.4		65	39.7	15.7	44.7	241	
13	44.5	6.33	44.0	79.1	Irregular	75	23.5	23.5	63.8	354	
17	43.6	9.09	39.7	89.5		76	25.4	8.60	54.2	215	
23	48.5	7.76	35.9	83.3		78	59.2	1.87	21.0	48.8	
27	66.3	12.7	22.5	492		82	26.2	8.56	65.6	788	3 ¹⁰ Helix
28	42.1	4.83	42.1	59.7	Helix B	83	29.0	9.53	52.5	282	
29	58.3	2.40	28.1	59.4		84	26.1	9.81	48.3	262	
31	36.9	4.39	48.6	68.7		92	29.6	3.54	56.6	52.8	
34	40.3	8.00	45.5	97.7	Irregular	93	34.9	9.11	35.5	76.0	Helix C
36	24.5	6.68	53.5	53.1		94	22.2	4.93	57.7	64.0	
37	19.4	11.5	56.8	475		95	28.8	3.29	61.0	63.5	
38	16.6	11.2	69.7	350		96	19.4	1.43	74.8	53.5	
39	18.2	9.33	56.1	296	Small β -sheet	97	32.7	7.08	51.7	61.6	3 ¹⁰ Helix
40	22.2	7.14	59.6	451		99	28.1	2.07	56.4	40.5	
42	26.5	8.76	66.0	531		108	34.9	3.73	47.5	87.5	
44	14.2	6.29	48.2	151		111	48.0	3.89	38.1	68.4	Helix D
50	24.0	10.6	40.4	188	Large β -sheet	112	28.2	3.04	47.9	49.8	
52	33.1	9.29	52.7	238		115	44.7	8.13	42.7	51.6	
53	25.6	8.80	62.4	349		123	46.7	3.42	35.0	65.2	
56	17.7	15.7	63.7	375		124	67.0	2.00	17.8	33.3	
58	17.8	16.8	67.4	392		125	43.1	4.95	40.4	108	

The time courses of change in proton occupancies were fitted to the sum of two exponentials of the form $y = A e^{(-K_1 t)} + B e^{(-K_2 t)} + C$ (where A and B are the fractional amplitudes of the two phases and K_1 and K_2 are their rate constants; C is the apparent fractional amplitude of a third phase too slow to be followed in our experiments). The last column gives the secondary structural context of each amide in the native state.

A and B, with kinetics indistinguishable from those of amides in the helices (Table 1).

It is clear that the α -domain acquires a stabilizing core when, in most molecules, the β -domain has not become fixed in a stable conformation. To investigate further the structure of the α -domain at this stage we have studied the protection of tryptophan indole NHs, two of which exchange slowly enough in the native enzyme to be used as probes of refolding²³. The kinetics of protection of Trp 111 resemble closely those of the main chain amides of the α -domain (Fig. 1). In the native structure this residue is in the D-helix and the side-chain NH is hydrogen bonded to the side-chain oxygen of Asn 27, in the B helix²⁴. Thus, its protection may monitor 'docking' of these two helices; at the very least it suggests that the tryptophan side-chain becomes excluded from solvent in a well developed hydrophobic core on this time scale. Similar behaviour was

observed qualitatively for the indole NH of Trp 28 which is also buried in the core of the α -domain.

The amide NH of Asn 27, which forms a non-helical hydrogen-bond to the carbonyl oxygen of Tyr 23 in the native structure, is the one marked exception to the general pattern of behaviour in the α -domain. Its protection kinetics resemble those of amides in the β -domain, with a slow phase time constant greater than 400 ms. This interaction may thus become fixed only late in folding, when both domains are organized. Therefore, although in most molecules the α -domain folds independently, at least some details of its tertiary interactions form more slowly.

Within the β -domain the situation is more complicated. With the exceptions of Trp 63, Cys 64 and Ile 78, there is a clear qualitative distinction between the protection kinetics of amides in this domain and in the α -domain but the slow phase time constants for individual amides vary between 150 and nearly

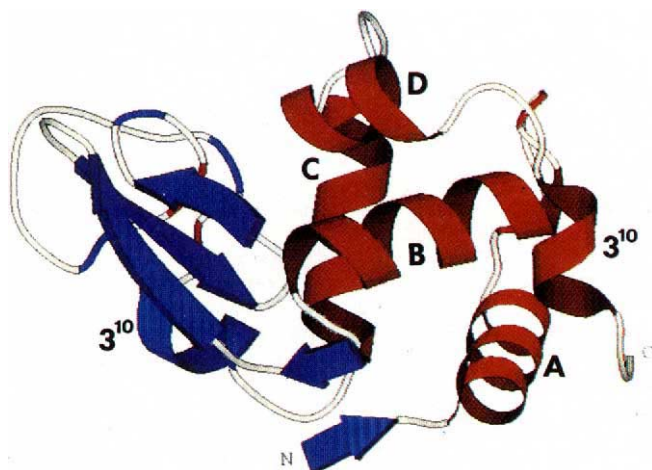


FIG. 2. Schematic view of the native structure of hen lysozyme. Elements of secondary structure in the α -domain are coloured red, as are three amides protected with similar kinetics, Trp 63, Cys 64 and Ile 78. Elements of secondary structure that are in the β -domain are coloured blue. White regions represent mainly surface amides for which there is no information. The four α helices (A–D) and the two 3¹⁰ helices are labelled. The diagram was produced using the program MolScript⁴³.

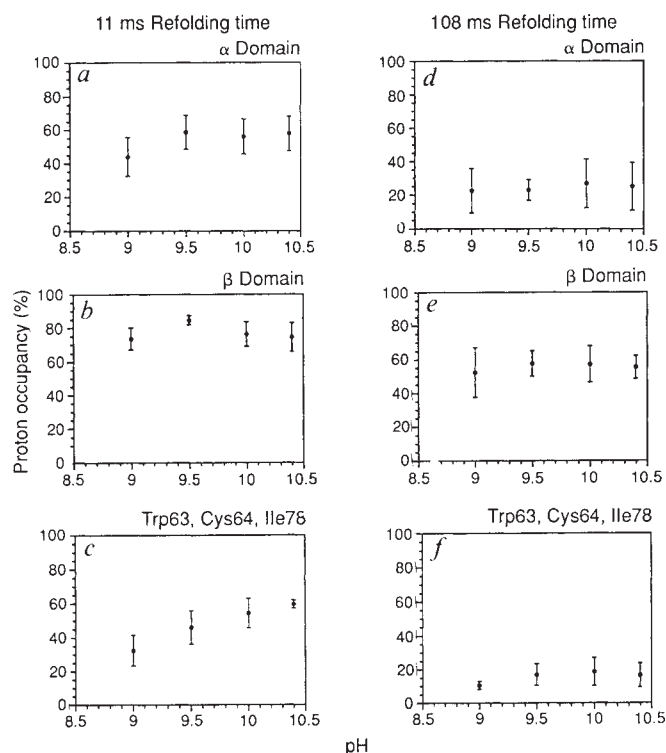


FIG. 3 The pH dependence of the extent of labelling during an 8.4 ms pulse commencing 11 ms and 108 ms after the initiation of folding. Exchange in proteins usually follows EX2 kinetics, in which the intrinsic rate of the chemical exchange step is slow compared with the rate of the structural fluctuations which expose the amide transiently to the solvent^{44,45}. In this situation, if the partial protection achieved in the fast phase reflects a single population of a marginally stable intermediate, then the extent of labelling would be predicted to increase substantially as the pH of the labelling pulse is raised^{27,39}. If, by contrast, the mechanism of exchange is EX1, where the overall rate is limited by the rate of the fluctuations that expose the amide to solvent water, the pH dependence of the extent of labelling, if any, would not be predictable in a straightforward manner. This ambiguity can be resolved by increasing the duration, rather than the pH, of the labelling pulse^{5,6}. This would result in increased labelling of any marginally stable intermediates regardless of the mechanism of exchange. In this figure the average proton occupancies for amide hydrogens in the α -domain (a, d), the β -domain (b, e) and the subset containing Trp 63, Cys 64 and Ile 78 (c, f) are shown as a function of the pulse pH. Vertical bars represent one standard deviation from the average plotted. The pH of the labelling pulse was varied between 9.0 and 10.4 by changing the pH of the exchange buffer. For these experiments the quench buffer was 1 M acetic acid in H₂O solution. Acquisition, processing and analysis of NMR spectra were done as described in the legend to Fig. 1. Control experiments in which a sample of the native protein (90% deuterated at amide sites) was exposed to the different labelling pulses showed no increase in proton occupancy at the 49 sites considered here, confirming that these amides are stable to exchange in the native enzyme under these conditions.

800 ms (Table 1). This implies that the stabilizing tertiary interactions in this domain are not formed in a single cooperative step but that a number of individual or sequential assembly processes are involved.

Protection of the three amides, Trp 63, Cys 64 and Ile 78, which lie in the long loop at the interface between the α - and β -domains cannot be rationalized by examination of the native structure; Cys 64 is not hydrogen bonded and Ile 78 forms only a long tertiary hydrogen-bond²⁴. Thus, their protection cannot be associated with native secondary structure but, presumably, with exclusion from solvent by surrounding side-chains^{25,26}. These interactions need not be native-like. There are two disulphide bridges close by (64–80 and 76–94), the latter linking the α - and β -domains. Early clustering of these residues in cooperation with formation of the α -domain core could explain the similarity in their protection kinetics.

Multiplicity of events in folding

There are two fundamentally different possible explanations for biphasic kinetics in the labelling experiment: the fast phase leads either to partial protection of a particular amide in all molecules or to complete protection but in only a proportion of molecules. The first case implies formation of a marginally stable intermediate, followed by slower transformation into a stable, presumably native-like, structure. The second possibility involves parallel pathways such that a proportion of the protein folds rapidly to a structure that completely protects the amide concerned from exchange, whereas the remainder follows a route in which protective structure is acquired more slowly.

These possibilities can be distinguished by varying the duration and the intensity of the labelling pulse^{5,6,27}. The results (Fig. 3) indicate that the labelling of the majority of amides in both domains is essentially independent of the pulse pH. In addition, no significant change in the extent of labelling occurs when the pulse length is doubled or trebled (at refolding times of 22 and 108 ms, respectively). These results are not compatible with a simple sequential folding mechanism; there must be parallel folding pathways, some leading to protection of individual amides more rapidly than others.

The only exceptions to the observed insensitivity to the labell-

ing conditions are the amides of Trp 63, Cys 64 and Ile 78 (Fig. 3). This suggests that rapidly formed structure protects these amides only partially, full protection being acquired in the slower phase. However, the sensitivity to the pulse conditions is weaker than expected for a single marginally stable species. Instead, there must be a heterogeneous population, with varying levels of protection.

Native and non-native structure

The sequence of events detected in the far-UV CD (225 nm) is characterized by at least three kinetic phases (Fig. 4). A large negative ellipticity is acquired within the dead time of the experiment (2 ms), so that at the earliest measurable time its magnitude is close to that observed for the native enzyme. The CD at this wavelength is generally dominated by peptide groups in helical structure, suggesting that an average helix content close to that of the native enzyme is achieved in this time. Contributions to the CD from aromatic groups and disulphides cannot, however, be ruled out²⁸.

There follows a remarkable further development of the dichroism at 225 nm, so that the average negative ellipticity becomes greater than that of the native state, reaching a maximum at around 80 ms. The kinetics of this phase do not coincide closely with any of the phases of exchange protection we have observed. It seems likely that the excursion in the CD reflects the transient formation of some kind of non-native interactions although we cannot rule out the possibility that it arises from asynchronous development of effects that tend to cancel out in the native protein spectrum. Return of the ellipticity to its value in the native state then occurs in a third phase ($\tau \sim 300$ ms) which is much slower than protection of α -helical amides but is comparable to the slow phase of protection in the β -domain.

CD experiments were also done in the near-UV region. The latter monitors aromatic groups in specific orientations fixed through tertiary interactions, so that spectra of denatured proteins, even relatively compact 'molten globule' states, are characterized by loss of nearly all intensity at these wavelengths^{17,18}. The time development at 289 nm is quite different from that in the far-UV (Fig. 4). There is virtually no change within the dead time of the experiment and the entire kinetic amplitude can be

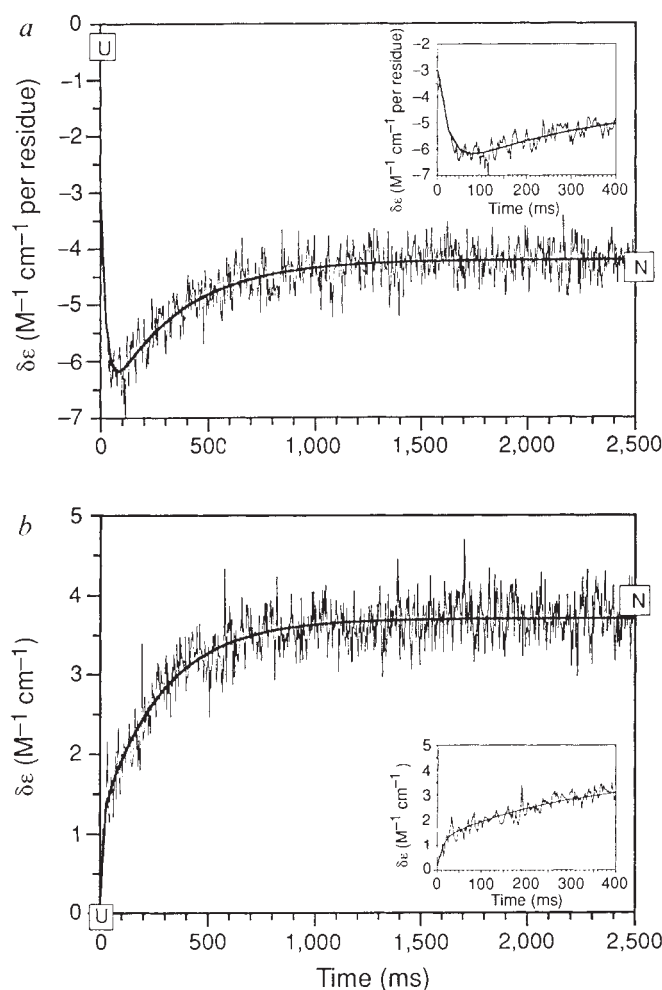


FIG. 4 Refolding kinetics of lysozyme as monitored by stopped flow CD at *a*, 225 and *b*, 289 nm. The refolding conditions were as described in the legend to Fig. 1, except that the final folding protein concentrations were 0.2 and 1.0 mg ml⁻¹, respectively. The experiments were done using a Jobin-Yvon CD6 circular dichrograph equipped with a Biologic SFM3 stopped-flow module. The dead time was about 2 ms. In each case the data have been fitted to a sum of two exponentials. The insets show an expansion of the plots during the first 400 ms of folding.

fitted to a sum of two exponentials, the amplitudes and time constants being $32 \pm 2\%$, $\tau = 10 \pm 2$ ms, and $68 \pm 2\%$, $\tau = 285 \pm 8$ ms. These parameters resemble those observed for protection of amides in the β -domain (Table 1).

The dichroism at 289 nm is associated principally with the six tryptophan residues of lysozyme²⁹. Of these, residues 62 and 63 are in an irregularly structured part of the β -domain, close to the domain interface, and the others are all in the α -domain. This is interesting in view of the apparent synchrony of their forming fixed tertiary contacts and the protection of amides in the β -, rather than the α -domain. Thus, although the α -domain can fold more rapidly than, and therefore independently of, the β -domain, the results suggest that its tertiary structure does not become fully organized until cooperative interactions spanning the two domains are established.

Folding pathways

The earliest event observed in refolding is the acquisition within 2 ms of a far-UV CD of similar intensity to that of the native protein. This almost certainly reflects formation of a large amount of α -helical structure, at least some of it presumably native-like, yet no protection from hydrogen exchange is apparent at this stage (Fig. 5). This suggests that although the

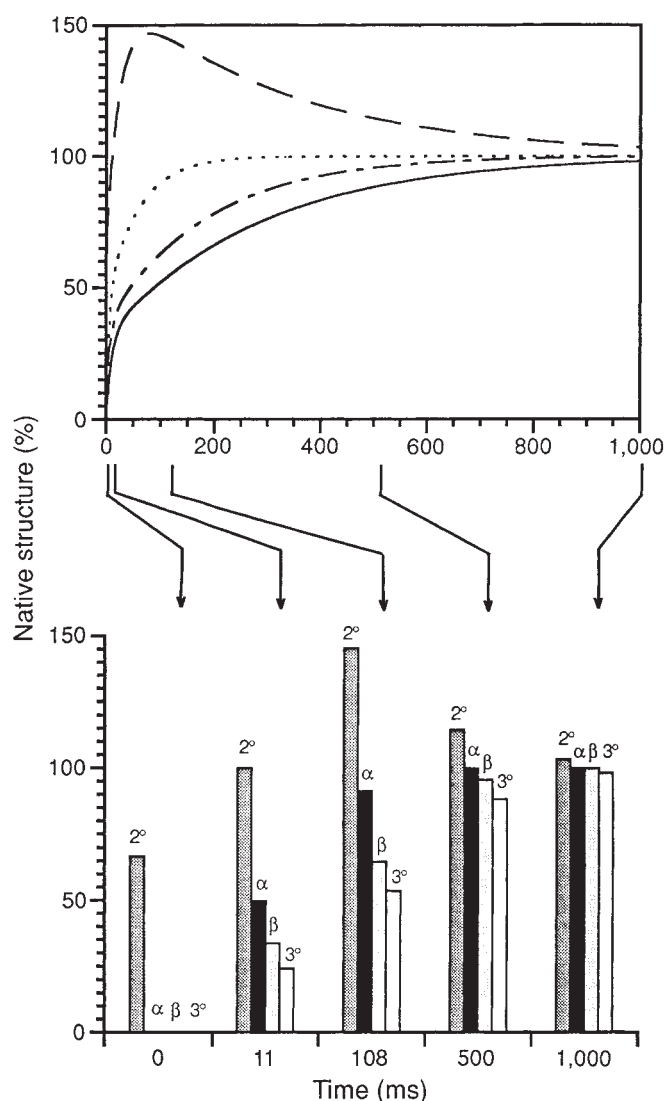


FIG. 5 Summary of events during the refolding of lysozyme. Formation of the native enzyme is virtually complete in 2 s (ignoring the very slow phase that was not recorded in our experiments) and each parameter is therefore assigned a value of 100% at this point. The curves shown are the fits to the near (289 nm; solid line) and the far (225 nm; dashed line) UV CD data and protection of the α - (dotted line) and the β -domains (dashed and dotted line), respectively. The latter two curves represent the averages of two exponential fits to the time courses for individual amides in each folding domain. For various different folding times the magnitude of each parameter as a percentage of that observed in the native protein is also shown: CD₂₂₅, Ellipticity at 225 nm; 3°, ellipticity at 289 nm; α , protection of amides in the α -domain; β , protection of amides in the β -domain.

average distribution of backbone conformations has become far from random, specific structural elements remain extremely labile. Similar behaviour has been observed in other early folding intermediates³⁰ and denatured states^{26,31,32}. This result is consistent with the near-UV CD which detects no specific interactions involving aromatic chromophores at this time. A change in the environment of these groups on this time scale has, however, been observed by stopped-flow absorption studies^{18,19}, suggesting that these rapid folding events include some kind of hydrophobic collapse as well as helix formation.

In the next phase there is rapid protection of α -domain amides in about 50%, and of β -domain amides in about 30%, of molecules. The protection data cannot reveal directly whether β -domain assembly occurs independently or in conjunction with that of the α -domain in individual molecules in this phase.

However, the presence of a phase with a similar time constant in the near-UV CD which, as discussed above, may reflect the formation of cooperative structure spanning both domains, suggests that roughly synchronous structure formation in both domains occurs in at least a substantial proportion of these molecules.

The remainder of the population folds more slowly and the greater amplitude of the fast phase and higher rate of the slower phase of protection for α -domain amides mean that in most molecules this domain becomes substantially folded before formation of a stable β -domain. In the resulting intermediates the pH dependence of labelling shows that for most amides in the α -domain the protection factors are in excess of 500 whereas those in the β -domain are less than 20. However, the slower development of near-UV CD intensity and of protection of at least one amide, Asn 27, in the α -domain suggest that it is not fully native-like but has some characteristics of a molten globule at this intermediate stage. The pattern of protection has some resemblance to that observed in the stable molten globule state of the homologous protein guinea-pig α -lactalbumin. In that case, fewer than 20 amides have protection factors greater than 10; again these occur in the α -, rather than the β -domain (C.-L. Chyan, C. Wormald, C.M.D., P.A.E. and J. Baum, manuscript in preparation). Similar, marginal protection is also characteristic of other stable partially folded proteins^{11,12,32}, suggesting that these are, in general, more labile structures than the kinetic intermediate observed in the present experiment.

The negative ellipticity in the far-UV CD, already substantially developed during the dead time, continues to grow during the first 80 ms or so of refolding, becoming substantially greater than that in the native enzyme spectrum. The origin of this signal cannot be determined from the present data but it is likely that it arises from some kind of non-native interactions. Reconstruction of CD spectra at these refolding times can provide further insight into this issue (M. E. Goldberg, personal communication). A similar overshoot has also been observed during the folding of the all- β protein, β -lactoglobulin³³. Return of the ellipticity towards its native value occurs at a similar rate to that of amide protection in the β -domain and the development of the near-UV CD; these are the slowest phases we observe (Fig. 5). This suggests that although the α -domain can fold to a large

extent independently, in most of the population the conformation of the remainder of the molecule, though not necessarily wholly disordered, is far from native-like until it becomes fixed in the cooperative tertiary structure.

A major unresolved issue is why different molecules fold by kinetically distinct pathways. This could reflect heterogeneity of the unfolded state^{20,34,35}; for example native-like X-Pro peptide bond isomers may be required for folding^{5,27,36-39}. It is possible that this could indeed account for the lack of protection in around 17% of molecules even after 2 s of refolding, but neither the rates nor amplitudes of the distinct phases resolved in our experiments are consistent with proline isomerization. The multiple pathways could reflect other heterogeneity in the denatured state or they may arise from an initial rapid collapse of the protein, leading to various intermediates, some amenable to further folding whereas others need first to reorganize, potentially a relatively slow process in a partially condensed state. A similar observation of parallel refolding pathways has been made for cytochrome *c* (ref. 40).

The four disulphide bridges persist in the denatured state and might have a significant influence on the refolding mechanism, either through their isomerism in the denatured state or in intermediates, or through constraints they place on conformational freedom during refolding. In α -lactalbumin persistence of substantial residual structure in its molten globule state is consistent with a number of different disulphide pairings⁴¹, suggesting that at least some intermediates can form independently of particular cross-links. Further experiments will be needed to assess the significance of disulphide bonds in the refolding of lysozyme.

These results have enabled us to develop a rather detailed picture of the folding pathway of hen lysozyme. CD and exchange labelling experiments have proved powerful and complementary structural probes, revealing, for example, the very rapid formation of helices before specifically stabilized interactions are detectable. The refolding kinetics are complex, in particular because there is evidently more than one folding pathway. An important general feature, not observed in other proteins studied by pulsed labelling methods^{5,6,21,42}, is the division of the protein into distinct folding domains which, in most molecules at least, are stabilized asynchronously. □

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Extreme-infrared brightness profile of the solar chromosphere obtained during the total eclipse of 1991

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THE solar chromosphere is a thin layer of gas that is several thousand degrees hotter than the underlying photosphere, and responsible for most of the Sun's ultraviolet emission. The mechanism by which it is heated to temperatures exceeding 10,000 K is not understood. Millimetre and submillimetre radiometry can be used to obtain the chromospheric temperature profile, but the diffraction-limited resolution for the largest telescopes is at best 17 arcsec, or $\sim 12,500$ km at the Sun's distance. This is greater than the thickness of the quiet chromosphere itself. The total eclipse of July 1991, which passed over the Mauna Kea Observatory in Hawaii, provided a rare opportunity to make limb occultation observations with a large submillimetre-wavelength telescope, the 15-m James Clerk Maxwell Telescope, and in this way we obtained a temperature profile in 1.3-mm radiation with ~ 300 km resolution at the Sun. Our observations indicate that spicules (magnetically entrained funnels of gas) reach a temperature of 8,000 K at 3,000–4,000 km above the photosphere, a temperature lower than those of many spicule models.

Limb occultation measurements at submillimetre wavelengths were first made in airborne eclipse observations on the Concorde¹ and the NASA Learjet², and later by the 1-m Kuiper Airborne Observatory^{3,4}, covering the spectrum from 30 to 670 μm . Remarkably, it is at 1.3 mm, where diffraction is most severe, that the James Clerk Maxwell Telescope (JCMT) has now given us the best measurement of this sort. We owe this to the improbable fortune of having a total solar eclipse over the world's best submillimetre telescopes, an accident that would be expected only once in ~ 400 years (ref. 5).

The JCMT is located at an elevation of 4,092 m near the summit of Mauna Kea. It is a Cassegrain design with a 15-m primary dish figured to the diffraction limit for 350- μm radiation. The resolution of the JCMT for 1.3-mm radiation is 22". For this observation we used the facility's millimetre-wave heterodyne receiver (receiver A) tuned to 230 GHz (wavelength 1.3 mm) with a passband of 500 MHz.

The observations were made on the morning of 1991 July 11. The Sun was at an elevation of 21° at mid-totality, appearing through three airmasses. Weather conditions for millimetre-wave observations were excellent. The eclipse started with first contact at 16:30:42 UT (06:30:42 local time, LT) and was already

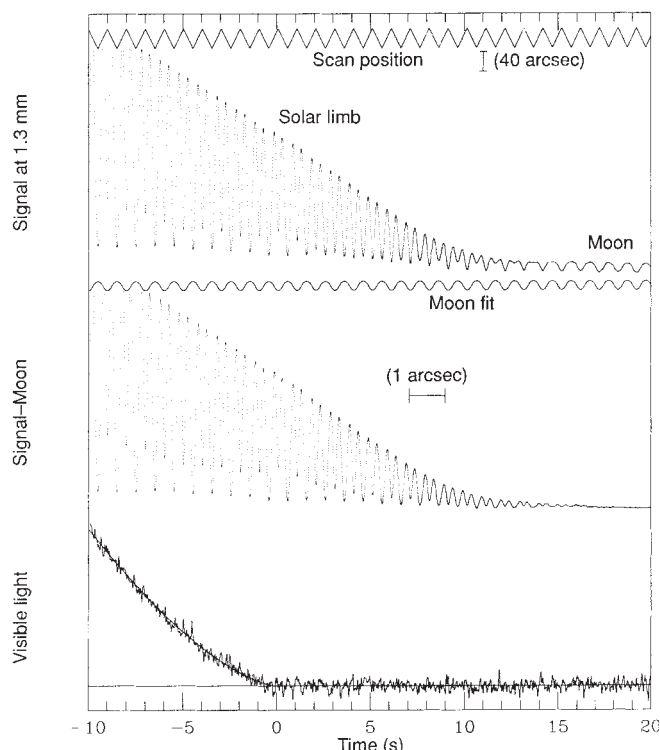


FIG. 1 Occultation of the extreme solar limb in 1.3-mm radiation, observed from the JCMT during the total solar eclipse of 1991 July 11 over Mauna Kea. The 20" beam of the JCMT scans across the disappearing solar crescent at the point of second contact for a distance of 40" twice each second in a triangular-wave mode, as indicated by the sawtooth profile (top). The 1.3-mm signal, sampled at 191.46 Hz, is shown in the profile just below, rapidly diminishing as the solar crescent is occulted, until only the signature of the Moon remains (extreme right). The lunar profile is extrapolated backwards from totality (see Moon fit) and subtracted from the occultation profile to give a representation of the solar occultation profile alone, shown below. The plot at the bottom shows the visible occultation, measured simultaneously from a heliostat outside the dome. The time of visible second contact, zero in this reference frame, is fixed (to within ± 0.15 s, equivalent to 0.07") by fitting the visible profile to a curve proportional to the 3/2 power of time before the occultation on top of the sky level, fitted to a constant during totality. Solar radiation at 1.3 mm is clearly seen more than 10 s after the visible limb has disappeared.

in progress when the JCMT acquired the Sun shortly after it cleared the summit cone $\sim 9^\circ$ above the horizon. Totality began with second contact (second contact is the moment at which the solar disk disappears behind the moon; third contact is the moment of re-emergence) at 17:28:09 UT (07:28:09 LT) and ended 4 minutes and 11 seconds later (third contact). The critical part of the observation occupied the few seconds before and after second and third contacts, the times during which the 20" beam of the telescope could encompass the width of the 1.3-mm solar crescent.

To observe the occultation, we used a rapid scanning procedure, driving the secondary reflector with a 1-Hz triangular wave to scan the receiver beam 40" across the solar crescent twice each second. This technique alleviates spurious signal variations from pointing errors (caused by atmospheric refraction) by forcing the beam across the solar crescent to sample its intensity at beam maximum once each scan. With this scheme we obtain a sampling interval of 0.26" as the occultation proceeds at $0.519^\circ \text{ s}^{-1}$. The occultation signals for second and third contacts are shown in Figs 1 and 2, respectively. The scan position is plotted with time at the tops of the figures. The resulting 1.3-mm signal is shown just below. As the solar crescent disappears, the signal from the solar limb rapidly diminishes, until only the signature of the lunar limb, with a brightness temperature of ~ 120 K, remains.

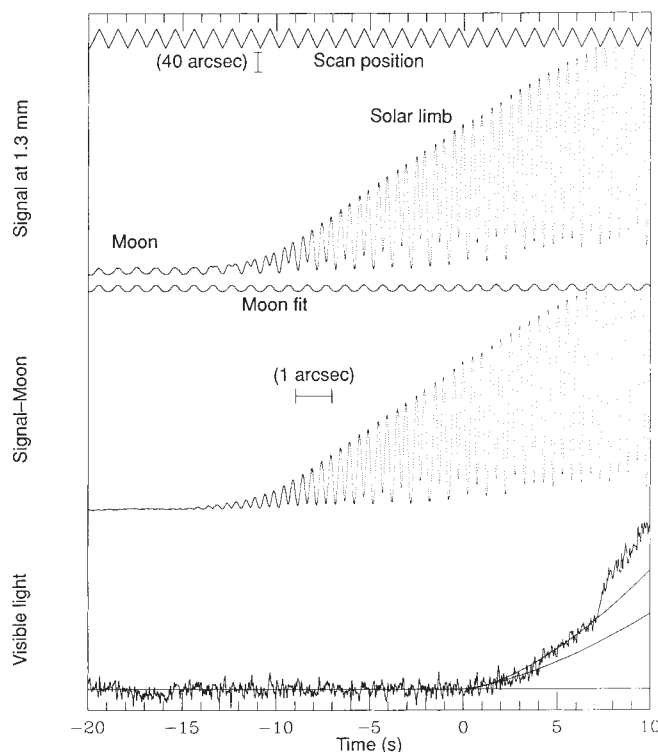


FIG. 2 Re-emergence of the 1.3-mm solar limb at third contact. This profile looks very similar to its second-contact counterpart. The main difference is a weaker lunar signature. The third-contact lunar surface, not having seen sunlight for up to two weeks, is noticeably cooler and proportionately dimmer than its early-evening counterpart at second contact.

We fit the lunar signature to a simple function ('Moon Fit' in both figures), which we extrapolate into the occultation to subtract it from the total signal. This gives us a representation of the solar occultation alone, shown below the Moon fit. The plots at the bottoms of Figs 1 and 2 show the visible occultation, measured by a silicon photocell at the focus of a 12.5-cm $f/24$ heliostat. The photocell was masked to encompass a 20° arc of the solar limb $10'$ long perpendicular to the limb at the point of contact. The timescales of Figs 1 and 2 are referenced at zero to the instant of visible contact. As a large amount of 1.3-mm radiation remains unocculted at the time of visible second contact, the 1.3-mm solar limb clearly extends far above the visible limb.

The basic procedure for reducing the occultation profiles to limb brightness profiles is described in refs 3, 4. Preliminary results of applying this procedure to the data in Figs 1 and 2 are shown in Fig. 3. When the solar crescent is much thinner than the beamwidth, the shape of the limb brightness profile is close to the simple derivative of the occultation curve. The top panel shows the limb brightness profile at second contact; the next panel shows that at third contact. The intensities are normalized to the brightness at disk centre. The extreme limb profiles measured at second and third contact are similar. They are $\sim 1.55 \pm 0.1$ times the brightness of quiet Sun at disk centre and extend $\sim 5''$ above the visible limb, with evidence of faint emission extending as far as $8''$. These limb extensions are significantly greater than the $4.2''$ extension found⁴ in 670- μm radiation from the Kuiper eclipse observations of 1988 March 17, in keeping with the increase in limb extension with increasing wavelength found throughout the submillimetre spectrum.

The uncertainty in the relative limb brightness is primarily due to the possibility of plage contamination of calibration scans made as the lunar silhouette passed near disk centre. Other

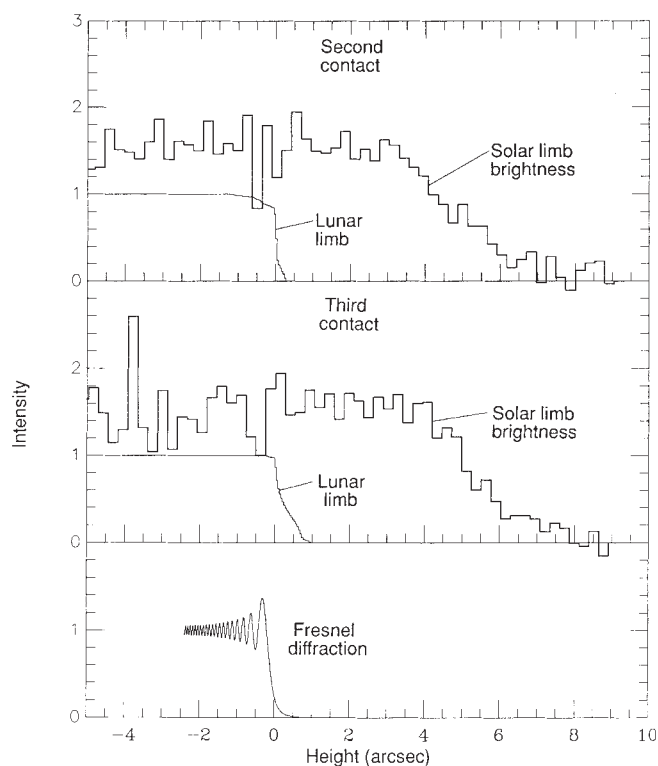


FIG. 3 Brightness profiles of the extreme solar limb in 1.3-mm radiation at the points of second and third contact calculated from the occultation signatures in Figs 1 and 2. On the ordinate scale adopted, unity is the brightness at disk centre. The resolution of the limb profile is limited mainly by Fresnel diffraction of solar radiation by the lunar limb, plotted in the bottom panel, and height variations of the lunar surface, due to mountains. Distributions of lunar heights in the second- and third-contact beams were convolved with a unit step function and plotted in the upper two panels under their respective solar limb profiles to represent the effects of lunar mountains.

sources of error include smearing of the occultation profile because of Fresnel diffraction of 1.3-mm radiation by the lunar limb, or by mountains on the lunar skyline. The effects of Fresnel diffraction on an idealized sharp limb of unit intensity are represented in the lower panel of Fig. 3. The effects of lunar mountains are similarly represented in the upper two panels below the solar limb brightness profiles. These distributions were derived from lunar limb plots calculated for our perspective by A. Fiala at the United States Naval Observatory. These errors will combine to smear a very sharp limb profile to a width of $\sim 0.4''$ (for the sharper second-contact lunar limb). This is small compared with the scale height and extension of the limb emission that we observe. We regard these qualities as entirely characteristic of the solar chromosphere itself. Although both limbs had activity nearby, and there was an intense active prominence near the point of third contact, which we mapped in detail (R.A.H., M.K.C., T.A.C., C.L., J.T.J., D. Sime, G.W., T.R., E.E.B., D.A.N., G.J.T. and D.C.B., manuscript in preparation), the regions of contact themselves were clear of activity. The limb heights at half intensity at second and third contact are equal to within $\sim 0.5''$.

$H\alpha$ photographs of the solar limb⁶⁻⁸ show a chromosphere characterized largely or entirely by spicules at the $5''$ height where the 1.3-mm limb appears. At about this height one begins to see individual spicules protruding above an underlying dense forest of rough structure. This suggests an optical depth of order unity for individual spicules at this height.

The 50% limb-brightening excess that we see at 1.3 mm is significant. It seems to be mostly localized, to within $15\text{--}20''$ of the visible limb. The relatively modest limb brightening seen at

submillimetre wavelengths^{3,4} has prompted us^{4,9} to model spicules as considerably cooler than previous models⁷ (only about 6,000 K). The 1.3-mm observations seem to indicate a substantial increase in temperature higher in the spicule, perhaps to 8,000 K at heights of 3,000–4,000 km, an important feature for consideration in spicule models. This higher temperature would still be substantially cooler than earlier spicule models at similar heights. □

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Linear free energy relations for predicting dissolution rates of solids

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A QUANTITATIVE understanding of the rates and mechanisms of dissolution of crystalline solids in aqueous solutions is critical to the chemical modelling of many geochemical, environmental and industrial processes. Here I show that a linear free energy equation, developed recently^{1,2} for the prediction of the standard Gibbs free energies of formation of isostructural families of crystalline solids, can also be used for predicting the dissolution rates of solids. This equation bears a close analogy with the Hammett equation for aqueous organics³. Regression of data for the surface-reaction-controlled dissolution rates of isostructural families of divalent metal oxides and orthosilicates using the new equation yields coefficients characteristic of the specific crystal structure, which

turn out to be very close to the coefficients obtained by regression of standard free energy data for the same families. These results suggest that standard free energy coefficients can be used to predict dissolution rates.

For any isostructural family of solids represented by M_vX (where M is a divalent cation, X represents the remainder of the formula of the solids, and v the number of moles of M per mole of solid), the free energy of formation of M_vX is given by the empirical equation^{1,2}

$$\Delta G_{f,M_vX}^0 = a_{M_vX} \Delta G_{n,M^{2+}}^0 + b_{M_vX} + \beta_{M_vX} r_{M^{2+}} \quad (1)$$

where a_{M_vX} , b_{M_vX} and β_{M_vX} are coefficients characteristic of the particular crystal structure of M_vX , and $r_{M^{2+}}$ is the radius of M in a given coordination state (Table 1). The parameter $\Delta G_{n,M^{2+}}^0$ for the aqueous cation M^{2+} (Table 1) is calculated from the standard Gibbs free energy of formation of M^{2+} ($\Delta G_{f,M^{2+}}^0$) using

$$\Delta G_{n,M^{2+}}^0 = \Delta G_{f,M^{2+}}^0 - \Delta G_{s,M^{2+}}^0 \quad (2)$$

where $\Delta G_{s,M^{2+}}^0$ is a free energy of solvation¹.

Although equation (1) applies to the free energies of crystalline solids, it is closely analogous to a family of linear free energy equations developed for aqueous and mixed-solvent

TABLE 1 Ionic radii and thermodynamic and kinetic data for aqueous cations and crystalline solids

M	$r_{M^{2+}}$ (Å)	ΔG_n $M^{2+}(\text{aq})$	ΔG_f				$\log r'$				$\Delta G_f^{\#}$			
			MO* (halite)	MO† (zincite)	M ₂ SiO ₄ ‡ (olivine)	M ₂ SiO ₄ † (phenacite)	MO§ (halite)	MO§ (zincite)	M ₂ SiO ₄ (olivine)	M ₂ SiO ₄ (phenacite)	MO (halite)	MO (zincite)	M ₂ SiO ₄ (olivine)	M ₂ SiO ₄ (phenacite)
Be	0.45	85.23		−138.60		−486.26		−14.2		−14.6		−119.2		−466.3
Mg	0.72	36.97	−136.04		−491.15		−7.5		−12.12		−125.8		−474.6	
Ca	1.00	−10.83	−144.2		−526.00		−6.9		−8.7		−134.7		−514.1	
Mn	0.82	81.26	−86.74		−389.94		−8.1		−9.4		−75.68		−377.1	
Fe	0.77	119.17			−329.86				−10.52				−315.5	
Co	0.735	131.35	−51.2		−312.09		−10.0		−11.21		−37.55		−296.7	
Ni	0.7	136.85	−50.6				−13.7				−31.90			
Cu	0.73	160.38												
Zn	0.745	108.23		−76.64		−365.165		−7.3		−9.49		−66.68		−352.2
Sr	1.16	−24.41	−137.12											
Cd	0.95	106.74												
Sn	1.11	106.28												
Ba	1.36	−36.73	−124.38											
Eu	1.17	−20.50	−133.1											
Hg	1.02	159.07												
Pb	1.18	102.10												
Ra	1.39	40.06												
UO ₂	0.754	−85.15												

Experimental ionic radii for divalent cations in crystalline solids ($r_{M^{2+}}$), values of the aqueous cation parameter ΔG_n from ref. 1, experimental values of the standard Gibbs free energies of formation (ΔG_f) of isostructural solids, and rates of dissolution of the solids. All free energy and rate values refer to 25 °C and 1 bar and are in kcal mol^{–1} and mol cm^{–2} s^{–1}, respectively. Values of the effective free energy of formation of surface complexes ($\Delta G_f^{\#}$) calculated with equations (12) and (13) are also in kcal mol^{–1}. Structure types of the solids are given in parentheses.

* Ref. 1.

† Ref. 15.

‡ Mg and Fe from ref. 16; Ca from ref. 17; Mn and Co from ref. 18.

§ Rates refer to pH 1.0 from ref. 8.

|| Rates refer to pH 2.0 from ref. 7.

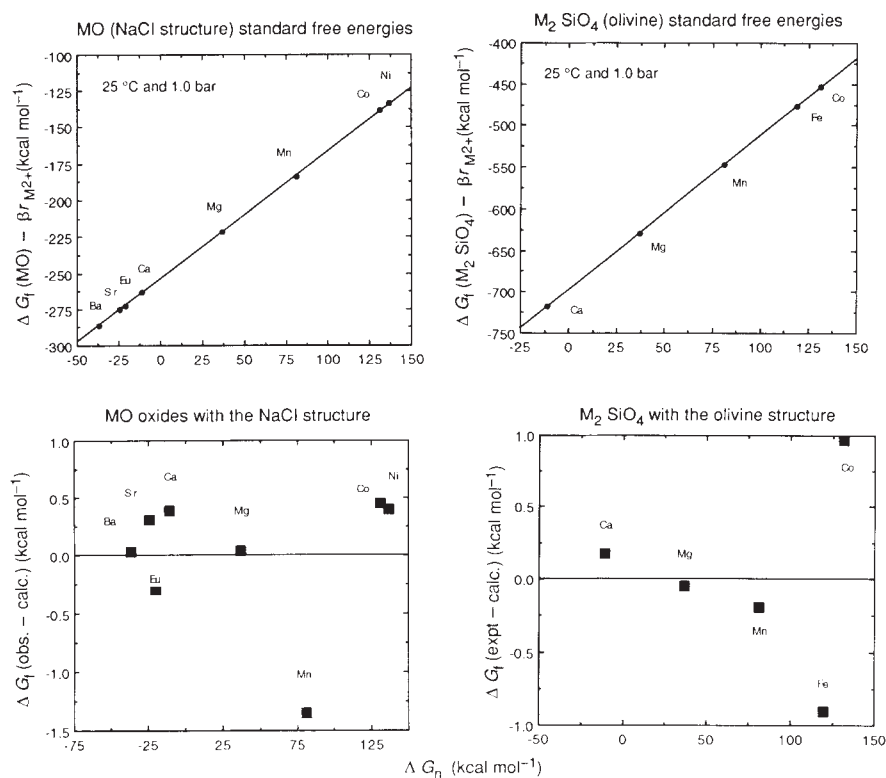


FIG. 1 Graphical representation of equation (9) for the standard Gibbs free energies of formation of metal oxides with the halite structure and orthosilicates with the olivine structure. Regression calculations were done using equation (1) and data from Table 1. The lower diagram in each case shows experimental minus calculated values. Values of the regression coefficients $a_{M,X}$, $b_{M,X}$ and $\beta_{M,X}$, and their standard errors, are summarized in Table 2a and were used to generate the standard free energies of formation listed in Table 2b. The elemental labels indicate M in the formula. With few exceptions, the agreement between experimental and calculated values is closer than 1.0 kcal mol⁻¹ and often less than 0.5 kcal mol⁻¹.

organic reactions³⁻⁵. One of these is the Hammett equation, which was originally developed to predict equilibrium and rate constants of substituted benzenes in solution⁶, and has the form

$$\log (K / K_0) = \sigma \rho \quad (3)$$

K and K_0 represent the equilibrium (or rate) constants for substituted and unsubstituted reactions involving a series of substituent functional groups, each of which has a characteristic parameter σ , and ρ is a regression coefficient characteristic of the reaction conditions. The substituent parameter was derived from the equilibrium constants of substituted and unsubstituted benzoic acids (K^b and K_0^b , respectively) according to the equation

$$\sigma = \log (K^b / K_0^b) \quad (4)$$

The relationship of equation (1) to the Hammett equation can be simply expressed by converting equation (1) to equilibrium constants and including a reference reaction for a specific cation N, to give

$$\log (K_{M,X} / K_{N,X}) = \sigma_{M^{2+}} \rho_{M,X} + \beta_{M,X} r^* \quad (5)$$

where

$$\sigma_{M^{2+}} = \log (K_{M^{2+}} / K_{N^{2+}}) \quad (6)$$

$$\rho_{M,X} = a_{M,X} \quad (7)$$

and

$$r^* = -(r_{M^{2+}} - r_{N^{2+}}) / 2.303 RT \quad (8)$$

Clearly, equation (5) is similar to equation (3), but the addition of $\beta_{M,X} r^*$ enables us to apply the new equation accurately to crystalline solids. Instead of quantifying the systematics of substituent functional groups on dissolved organic species, equations (1) and (5) quantify the systematics of cation substitution in crystalline solids.

Table 1 summarizes standard free energy and rate data for metal oxides (MO) with the halite and zincite structures, and for orthosilicates (M_2SiO_4) with the olivine and phenacite structures.

A linear free energy correlation for the standard Gibbs free energies of the halite-type oxides has already been reported¹ (Table 2a). For the olivine structure, the standard Gibbs free energy data in Table 1 were regressed using equation (1) to give the parameters in Table 2a. From these results and those for polymorphic fluoride and carbonate structures¹, it can be assumed that oxides with the halite and zincite structures have the same a_{MO} parameter, and similarly, that the olivines and phenacites have the same $a_{M_2SiO_4}$ parameter. I therefore regressed the remaining pairs of standard Gibbs free energies in Table 1 using the values of a_{MO} and $a_{M_2SiO_4}$ established above to generate values of $b_{M,X}$ and $\beta_{M,X}$ for the zincite and phenacite structures (Table 2a). Calculated values of the Gibbs free energies for 76 solids from all four isostructural families are listed in Table 2b.

The strong linear free energy correlation for the halite-type metal oxides and the olivines can be seen graphically by

TABLE 2a Regression parameters

Isostructural type (M_vX)	$a_{M,X}$	$b_{M,X}$ (kcal mol ⁻¹)	$\beta_{M,X}$ (kcal mol ⁻¹ Å ⁻¹)
MO (halite)	0.8756 (0.0076)	-254.21 (2.34)	119.10 (2.17)
MO (zincite)	0.8756	-277.02	141.75
M_2SiO_4 (olivine)	1.8559 (0.011)	-697.68 (5.40)	191.60 (5.91)
M_2SiO_4 (phenacite)	1.8559	-764.04	265.77
	$a_{M,X}^{\#}$	$b_{M,X}^{\#}$ (kcal mol ⁻¹)	$\beta_{M,X}^{\#}$ (kcal mol ⁻¹ Å ⁻¹)
MO (halite)	0.9041 (0.031)	-246.02 (13.4)	120.42 (14.6)
MO (zincite)	0.8756	-243.28	109.83
M_2SiO_4 (olivine)	1.8412 (0.018)	-666.28 (8.49)	171.61 (9.29)
M_2SiO_4 (phenacite)	1.8559	-733.47	242.12

Summary of regression parameters from equations (1) and (14) for four families of isostructural solids (standard errors are given in parentheses). All values refer to 25 °C and 1 bar.

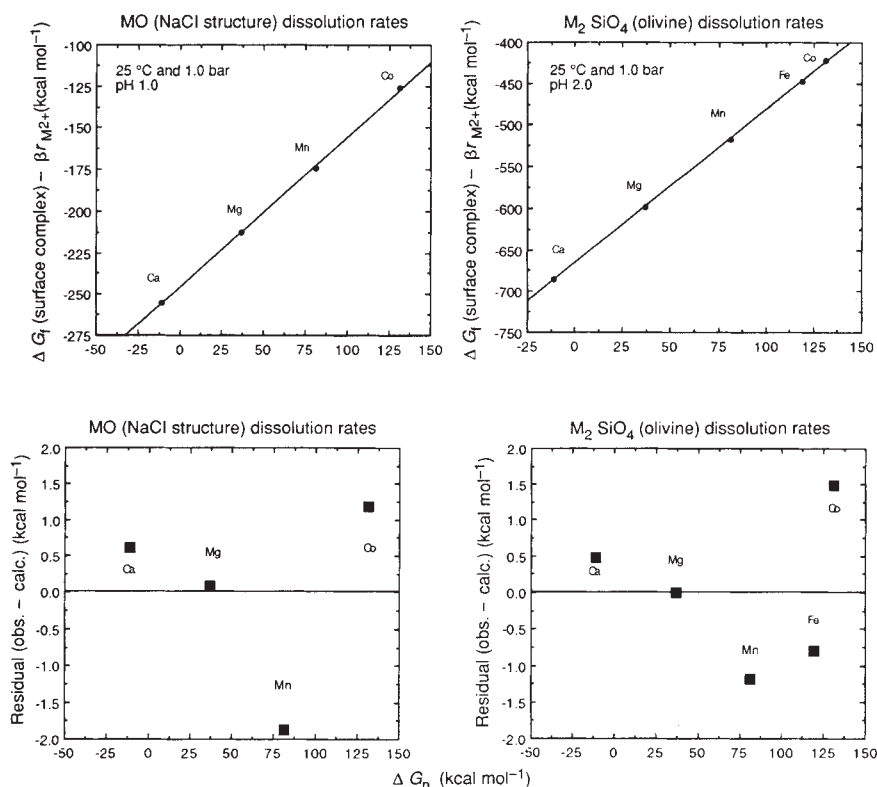


FIG. 2 Graphical representation of equation (15) for the effective free energies of formation of surface complexes on metal oxides with the halite structure and orthosilicates with the olivine structure. Regression calculations were carried out with equation (14) and data from Table 1. The lower diagram in each case shows experimental minus calculated values. Values of the regression coefficients $a_{M,X}^{\#}$, $b_{M,X}^{\#}$ and $\beta_{M,X}^{\#}$, and their standard errors, are summarized in Table 2a and were used to generate the effective free energies and the predicted rates of dissolution listed in Table 2b. Elemental labels indicate M in the formula. With few exceptions, the agreement between experimental and calculated values is closer than $1.0 \text{ kcal mol}^{-1}$.

rearranging equation (1) into the form

$$\Delta G_{f,M,X}^0 - \beta_{M,X} r_{M^{2+}} = a_{M,X} \Delta G_{n,M^{2+}}^0 + b_{M,X} \quad (9)$$

and plotting calculated values of the function $\Delta G_{f,M,X}^0 - \beta_{M,X} r_{M^{2+}}$ versus the aqueous cation parameter $\Delta G_{n,M^{2+}}^0$ (Fig. 1). Experimental minus calculated values from Tables 1 and 2b are also given in Fig. 1. Clearly, equation (9) provides an excellent fit to the data, within $1.0 \text{ kcal mol}^{-1}$, as found for numerous other crystalline solids^{1,2}.

The success of equation (1) in describing the standard Gibbs free energies of formation of numerous isostructural families^{1,2} of crystalline solids, and the analogy with the Hammett equation

discussed above, suggests that linear free energy relations for crystalline solids might also be applicable to the rates at which solids react. Here I explore this possibility for isostructural families of divalent metal oxides and silicates for which the experimental surface-reaction controlled dissolution rates under uniform (acidic) conditions have been discussed recently^{7,8}.

According to the hypothesis of surface-reaction control and transition state theory⁹⁻¹¹, the rate of reaction for an oxide or silicate (r') far from equilibrium can be related to the rate constant (k') by the equation

$$\log r' = \log k' + n' \log (a_{H^+}) \quad (10)$$

TABLE 2b Predicted free energies of formation and rates of dissolution

M	ΔG_f^0				$\Delta G_f^{\#}$				$\log r'$			
	MO (halite)	MO (zincite)	M ₂ SiO ₄ (olivine)	M ₂ SiO ₄ (phenacite)	MO (halite)	MO (zincite)	M ₂ SiO ₄ (olivine)	M ₂ SiO ₄ (phenacite)	MO (halite)	MO (zincite)	M ₂ SiO ₄ (olivine)	M ₂ SiO ₄ (phenacite)
Be	-125.98	-138.60	-453.27	-486.26	-114.7	-119.22	-432.13	-466.34	-8.2	-14	-15.5	-14
Mg	-136.08	-142.57	-491.10	-504.05	-125.8	-131.82	-474.64	-490.52	-7.4	-7.8	-12.0	-9.9
Ca	-144.59	-144.74	-526.17	-518.36	-135.3	-142.92	-514.61	-511.45	-6.7	-1.3	-8.47	-5.0
Mn	-85.39	-89.62	-389.74	-395.28	-73.80	-82.056	-375.93	-384.11	-8.4	-5.5	-10.1	-8.1
Fe	-58.15	-63.51	-328.95	-338.20	-45.54	-54.352	-314.71	-325.85	-9.2	-6.7	-10.4	-9.0
Co	-51.65	-57.81	-313.05	-324.90	-38.75	-47.531	-298.28	-311.72	-9.4	-7.5	-10.8	-9.6
Ni	-51.00	-57.95	-309.55	-323.99	-37.99	-46.558	-294.16	-309.98	-9.5	-8.3	-11.2	-10
Cu	-26.83	-33.10	-260.13	-272.35	-13.10	-22.661	-245.69	-259.05	-10	-7.6	-10.5	-9.7
Zn	-70.71	-76.64	-354.05	-365.16	-58.45	-66.681	-339.15	-352.22	-8.9	-7.3	-10.9	-9.4
Sr	-137.43	-133.95	-520.72	-501.04	-128.4	-137.24	-512.16	-497.92	-6.6	2.41	-6.27	-2.2
Cd	-47.60	-48.89	-317.55	-313.45	-35.11	-45.472	-306.72	-305.35	-9.1	-2.5	-7.93	-5.9
Sn	-28.95	-26.61	-287.74	-271.77	-16.26	-28.299	-280.10	-267.46	-9.2	1.24	-5.59	-3.1
Ba	-124.40	-116.39	-505.27	-470.75	-115.4	-126.06	-500.53	-472.36	-6.5	7.09	-3.47	1.17
Eu	-132.81	-129.11	-511.55	-491.12	-123.6	-132.72	-503.24	-488.23	-6.7	2.64	-6.08	-2.1
Hg	6.56	6.86	-207.00	-197.71	20.631	8.04311	-198.34	-191.27	-10	-0.86	-6.34	-4.7
Pb	-24.27	-20.34	-282.08	-260.92	-11.61	-24.267	-275.78	-258.26	-9.2	2.87	-4.61	-1.9
Ra	-123.74	-115.05	-505.70	-468.95	-114.8	-125.68	-501.50	-471.27	-6.5	7.79	-3.07	1.69
UO ₂	-238.96	-244.69	-711.24	-721.67	-232.2	-235.01	-693.66	-708.94	-4.9	-7.0	-12.8	-9.3

The standard Gibbs free energies of formation (ΔG_f^0) and effective Gibbs free energies of formation of the surface complex ($\Delta G_f^{\#}$), in kcal mol^{-1} , were calculated using equations (1) and (14) and the regression parameters given in Table 2a. Rates of formation were calculated from the effective free energies ($\Delta G_f^{\#}$) using equations (12) and (13), and refer to pH1 for MO solids and pH2 for M₂SiO₄ solids.

where a_{H^+} represents the activity of the aqueous H^+ ion and n' the order of the reaction⁹ (see refs 12–14 for surface complexation theory). For the purposes of defining linear free energy correlations, the critical feature of the data in Table 1 is that the dissolution rates refer to a constant pH for each isostructural family. Because the value of n' for each structure is roughly constant^{7–9}, it is possible to develop a number of linear free energy relations involving the function $2.303RT \log r'$. One such relation employs the cation parameter $\Delta G_{s,M^{2+}}^0$ according to

$$2.303RT \log r' + \beta r_{M^{2+}} = a' \Delta G_{s,M^{2+}}^0 + b' \quad (11)$$

which yields strong linear free energy correlations, using the β values defined by the regression of the standard Gibbs free energies.

An even more direct analogy with the standard free energies of formation in equation (1) can be developed by defining an empirical effective free energy of reaction ($\Delta G_r^\#$) forming a surface complex^{9–14} on the surface of a solid according to

$$\Delta G_r^\# = -2.303RT \log r' \quad (12)$$

and assuming further that the formation of the surface complex involves only the addition of H^+ ions to the surface of the solid^{9–14}, which permits calculation of an effective free energy of formation of the surface complex ($\Delta G_f^\#$) from

$$\Delta G_f^\# = \Delta G_r^\# + \Delta G_{f,M^{2+}}^0 + n' \Delta G_{f,H^+}^0 \quad (13)$$

I assume here that n' is a constant and that $\Delta G_{f,H^+}^0$ can be represented by the conventional apparent free energy of formation of the hydrogen ion. Definition of this empirical free energy of formation ($\Delta G_f^\#$) permits the most direct analogy to equation (1) through the following linear relation

$$\Delta G_{f,M^{2+}}^\# = a_{M^{2+}}^\# \Delta G_{n,M^{2+}}^0 + b_{M^{2+}}^\# + \beta_{M^{2+}}^\# r_{M^{2+}} \quad (14)$$

where the parameters $a_{M^{2+}}^\#$, $b_{M^{2+}}^\#$ and $\beta_{M^{2+}}^\#$ are regression parameters analogous to the parameters $a_{M^{2+}}$, $b_{M^{2+}}$ and $\beta_{M^{2+}}$ in equation (1). Values of $\Delta G_f^\#$ listed in Table 1 were calculated using equations (12) and (13) and the experimental values of $\Delta G_{f,M^{2+}}^0$ and rates given in Table 1. Regression of the $\Delta G_f^\#$ values of the metal oxides with the halite structure and the orthosilicates with the olivine structure using equation (14) yielded the parameters $a_{M^{2+}}^\#$, $b_{M^{2+}}^\#$ and $\beta_{M^{2+}}^\#$ summarized in Table 2a and the calculated values of $\Delta G_f^\#$ in Table 2b. Rearrangement of equation (14) leads to

$$\Delta G_{f,M^{2+}}^\# - \beta_{M^{2+}}^\# r_{M^{2+}} = a_{M^{2+}}^\# \Delta G_{n,M^{2+}}^0 + b_{M^{2+}}^\# \quad (15)$$

which is analogous to equation (9). This permits graphical depiction of the data and the calculated values for metal oxides and olivines in Fig. 2, which is closely analogous to Fig. 1. Clearly, equation (14) allows a very close fit to the experimentally derived rate data. Only the rate data for NiO were omitted from the regression calculations. Measured rates of dissolution of NiO range over at least two log units because of differences in the mode of annealing during high-temperature synthesis of the compound⁸. Most of the differences between experimental and calculated effective free energies in Fig. 2 are $\sim 1.0 \text{ kcal mol}^{-1}$ or less. On the basis of these fits, I used the calculated effective free energies to predict the rates of reaction of the 36 crystalline solids with the halite and olivine structures in Table 2b. In addition, by analogy with the approach taken above for the standard free energies, I used the values of $a_{M^{2+}}^\#$ generated above to generate further values of $b_{M^{2+}}^\#$ and $\beta_{M^{2+}}^\#$ for the zincite oxides and the phenacite silicates for each of which there are only two measured rates (Table 2a). Predicted effective free energies and corresponding rates of dissolution for the 36 crystalline solids with the zincite and phenacite structures are summarized in Table 2b.

Finally, the regression parameters summarized in Table 2a show that, within experimental uncertainties, the standard-state free energy parameter $a_{M^{2+}}^\#$ is remarkably close to the dissolution rate parameter $a_{M^{2+}}^\#$ for metal oxides with the halite structure

and for orthosilicates with the olivine structure. Similar considerations apply to $\beta_{M^{2+}}^\#$ and $\beta_{M^{2+}}^\#$. These results considerably enhance the predictive power of equations (1) and (14). They suggest that the free energy parameters $a_{M^{2+}}^\#$ and $\beta_{M^{2+}}^\#$ can be used in equation (14) to predict rates. Consequently, only one independently measured rate is needed for an isostructural family of solids. Predictive relations for crystalline solids in general are currently being explored further (D.A.S., manuscript in preparation). Such relations confirm the intriguing analogy between the Hammett approach for aqueous organics and the new equation for crystalline solids presented here. □

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Slowing down of the global accumulation of atmospheric methane during the 1980s

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MEASUREMENTS of methane in modern air^{1–8} and in air trapped in ice cores^{9–12} have shown convincingly that the abundance of atmospheric methane has been rising since the Industrial Revolution. This is a matter of concern because of the important role of methane in determining the radiative balance and chemical composition of the atmosphere¹³. The causes of this increase have not been identified unambiguously because of uncertainties in our understanding of the global budget of atmospheric methane¹⁴ and in how it is changing with time. Here we report on measurements of atmospheric methane from an extensive global network of flask sampling sites, which reveal that, although methane continues to accumulate in the atmosphere, there has been a substantial slowing of the global accumulation rate between 1983 and 1990. If this deceleration continues steadily, global methane concentrations will reach a maximum around the year 2006. Our results hint that changes in methane emissions in the latitude band 30–90° N may be of particular significance to this trend.

Our data set is based on $\sim 10,000$ air samples collected between May 1983 and December 1990, through the global

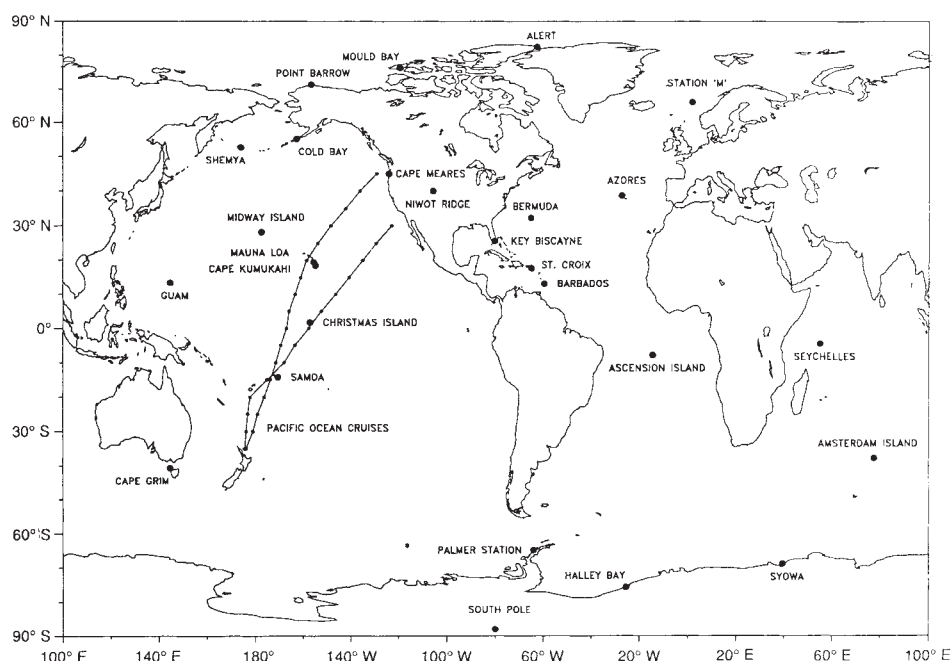


FIG. 1 Locations of the sampling sites in the NOAA/CMDL cooperative flask sampling network at the end of 1990. The 28 fixed sites are shown as large solid circles with their site names. Details on these sites are provided elsewhere^{4,15,16}. The shipboard sampling sites are indicated by smaller solid circles connected by straight line segments, located at intervals of 5° latitude between 45°N and 35°S. Shipboard samples are collected by officers of the Blue Star Line, Ltd container ship *M/V Southland Star*. Depending on ports of call, each crossing roughly follows one of the two tracks indicated. Data from each 5° latitude collection, regardless of track, are considered part of a single site, providing another 17 sites to the network.

cooperative flask sampling network (Fig. 1) operated by NOAA's Climate Monitoring and Diagnostics Laboratory (CMDL). Fixed sites are sampled roughly weekly, and shipboard samples are collected roughly every 3 weeks. The data set is dominated by samples representative of the marine boundary layer at locations remote from known or suspected methane source regions¹⁴. Methane (CH_4) is analysed by gas chromatography with flame ionization detection at the NOAA/CMDL facilities in Boulder, Colorado. Full details of the procedures and data can be found elsewhere^{4,15-17}.

Surface methane values at 14-day intervals averaged over global, hemispheric and semi-hemispheric latitude zones are generated from surface-area weighting of the spatially and temporally smoothed distribution of CH_4 shown in Fig. 2. The time series of the global and hemispheric values are shown in Fig. 3a. The main features of the data set are the strong latitudinal gradient with higher values in the Northern Hemisphere, the seasonal variation in both hemispheres and the long-term increase. The global average values show a seasonal variation^{4,8}, which reflects the difference between the seasonal cycles of the Northern and Southern Hemispheres. If the surface measurements accurately depict the global burden, the global burden of atmospheric CH_4 undergoes an annual cycle with a peak-to-peak amplitude of ~15 parts per 10^9 by volume in dry air (p.p.b.), or 43 Tg (1 Tg = 10^{12} g). Modelling studies¹⁴ suggest that the regular seasonal cycle of CH_4 in the Southern

Hemisphere is caused principally by the seasonality in the photochemical oxidation of CH_4 by hydroxyl radical (OH). The more complex nature of the CH_4 seasonality in the Northern Hemisphere arises through the interaction between seasonal sources and sinks.

To examine features of the growth rate for the data shown in Fig. 3a, as well as the semi-hemispheric data sets, we fitted to the data functions of the form

$$f(t) = a_1 + a_2 t + a_3 t^2 + \sum_{i=1}^4 (a_{2i+2} \sin(2\pi i t) + a_{2i+3} \cos(2\pi i t)) \quad (1)$$

which approximate the average long-term trend and average seasonal cycle. Coefficients from these fits for the seven data sets are given in Table 1. To account for interannual variations in the trend, the residuals are smoothed by multiplying them by a convolution function with a full width at half maximum (FWHM) of ~1 year (see ref. 19), and this curve is then added to the quadratic part of equation (1) to obtain a function $F(t)$ (Fig. 3a). The derivative of $F(t)$ (Fig. 3b) describes how the CH_4 growth rate has varied with time. Growth-rate curves produced from the data using different smoothing methods are nearly identical to those presented here. Probably the largest uncertainty in the calculated global growth rate is due to the number and distribution of the sampling sites used in the compilation of the global average CH_4 values. This has been evaluated

TABLE 1 Coefficients obtained for least-squares fits of equation (1) to the biweekly CH_4 mixing ratios

Latitude zone	a_1 (p.p.b.) (0.27)	a_2 (p.p.b. yr ⁻¹) (0.15)	a_3 (p.p.b. yr ⁻²) (0.02)	a_4 (p.p.b.) (0.10)	a_5 (p.p.b.) (0.10)	a_6 (p.p.b.) (0.10)	a_7 (p.p.b.) (0.10)	a_8 (p.p.b.) (0.10)	a_9 (p.p.b.) (0.10)	a_{10} (p.p.b.) (0.10)	a_{11} (p.p.b.) (0.10)
Global	1656.56	11.74	-0.31	-0.66	4.73	-2.33	-2.67	1.00	0.45	0.08	0.76
NH (0°-90°N)	1702.19	12.10	-0.41	6.87	13.36	-4.40	-5.11	2.05	1.04	0.01	1.68
SH (0°-90°S)	1610.91	11.39	-0.22	-8.18	-3.89	-0.26	-0.23	-0.06	-0.12	0.13	-0.16
HNH (30°-90°N)	1734.03	12.65	-0.58	5.12	15.72	-5.37	-7.99	3.53	1.76	-0.03	2.36
LNH (0°-30°N)	1670.35	11.54	-0.23	8.61	11.00	-3.43	-2.22	0.56	0.24	0.06	1.00
LSH (0°-30°S)	1615.69	11.17	-0.21	-3.33	-2.55	1.17	-0.40	0.25	-0.34	0.35	-0.24
HSN (30°-90°S)	1606.14	11.60	-0.22	-13.02	-5.23	-1.70	-0.04	-0.37	0.10	-0.08	-0.06

Time, t , is in years with $t=0$ at 1 January 1987. Values in parentheses are one standard deviation. HNH and LNH, high and low Northern Hemisphere; HSH and LSH, high and low Southern Hemisphere.

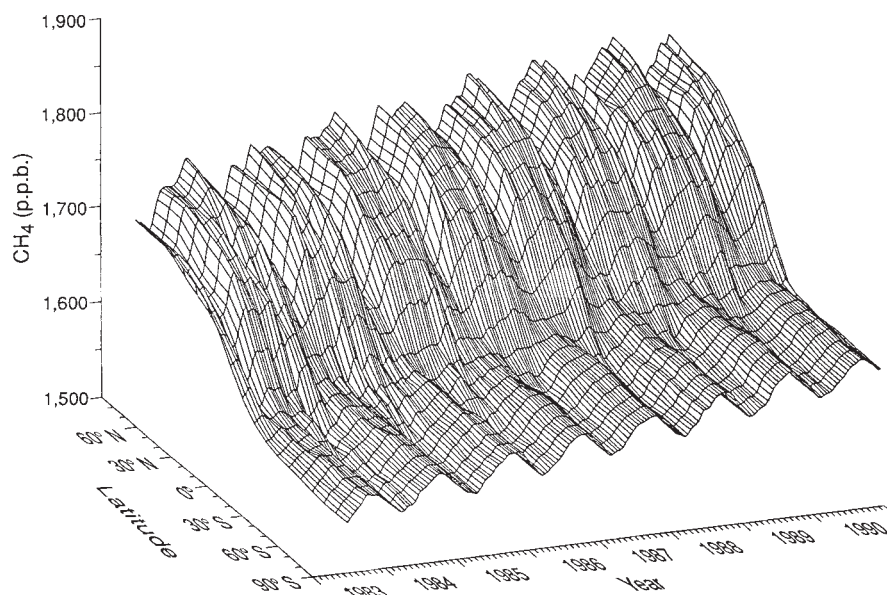


FIG. 2 Three-dimensional representation of the global distribution of atmospheric methane in the marine boundary layer, assuming no variation with longitude. Data from 37 sites are used. Grid spacing is 14 days by 10° in latitude. Global data began in May 1983. To generate this distribution, the data from each site are first fitted with a function of the type in equation (1), and the residuals filtered (smoothed by multiplying them by a convolution function with a FWHM of 40 days, see text). Curves resulting from the filtering are added to equation (1) to obtain the final description, from which biweekly values are taken, synchronized in time for all sites. A smooth curve as a function of latitude was drawn through each set of biweekly values using a digital filtering technique¹⁸. Data from Mauna Loa, Hawaii, and Niwot Ridge, Colorado, were omitted because the high altitude of these sites leads to lower methane mixing ratios than found in the marine boundary layer^{4,14}. Because of sparseness of data or gaps in the record, data from a further seven sites were not used (Halley Bay, Syowa, Seychelles, Azores and three shipboard sites at 45°N , 40°N , and 35°N). Even though it is not part of the sampling network, we included data from McMurdo, Antarctica (78°S , 167°E), covering the period December 1985 to October 1987.

with jack-knife and bootstrap statistical methods. In the jack-knife method²⁰, the entire growth-rate analysis was repeated 37 times (the number of sites used to generate the global data set), with the data from a single site removed in each analysis. The dependence of the globally averaged growth rate on data from any one site is small (Fig. 3b).

To address the uncertainties associated with the network distribution itself, we used a bootstrap method²⁰ in which global data sets were constructed by random selection of sites, with restitution, from the 37 sites used in generating the original global description. Data from sites could be represented more than once, or not at all. To be accepted, each bootstrap sample contained at least one Antarctic, one Arctic and one southern tropical site. We formed 10 bootstrap samples and calculated the globally averaged growth rates for each (Fig. 3b). We believe that this is a more realistic evaluation of the uncertainty of the globally averaged CH_4 growth rate, because the locations of the flask sampling sites depend on such factors as the existence of remote islands, personal contacts and logistics. The relatively long atmospheric lifetime of CH_4 and the large number of sampling sites means that our estimates of globally averaged growth rates should be largely insensitive to the addition of further sampling sites. The most compelling feature of the global CH_4 growth rate over this period is the overall deceleration, from $13.3 \text{ p.p.b. yr}^{-1}$ in 1983 to $9.5 \text{ p.p.b. yr}^{-1}$ in 1990. Decelerations have been reported previously for CH_4 records over different periods at individual sites in the Southern Hemisphere^{4,21}.

The bootstrap analysis also suggests that the only significant interannual variation in the globally averaged CH_4 growth rate is that for 1988–90. The growth-rate fluctuations seen here are smaller than those reported in other global data sets⁸. We believe that this is partly due to the much larger number of sampling sites used in our global record. Similar growth-rate analysis of records from all our individual sites, particularly Northern Hemisphere sites, indicates that they can show apparent growth-rate fluctuations much larger than seen in the global record.

The average CH_4 growth rate between 1984 and 1990 was $11.8 \text{ p.p.b. yr}^{-1}$ in the Northern Hemisphere and $10.9 \text{ p.p.b. yr}^{-1}$ in the Southern Hemisphere. The higher average growth rate in the Northern Hemisphere is consistent with an overall increase in the interhemispheric difference which increased from 87.3 p.p.b. in 1984 to 93.4 p.p.b. in 1987 (with an uncertainty

estimated from the bootstrap technique as $\pm 1 \text{ p.p.b.}$). Between 1987 and 1990, the interhemispheric difference stopped increasing, during which time the average growth rate was identical for both hemispheres at $10.3\text{--}10.4 \text{ p.p.b. yr}^{-1}$. Figure 3c shows the growth rate as a function of time for each hemisphere. Each decreases over the period of the measurements. The most striking feature of the growth rate in the Southern Hemisphere is the swing during 1988–89, which is closely matched by an anomaly of the opposite sign in the Northern Hemisphere. The changes in the Northern Hemisphere precede those in the Southern Hemisphere by about 4 months and are slightly smaller in amplitude. The annual interhemispheric difference was anomalously low in 1989. These CH_4 growth-rate anomalies correlate well with the La Niña event of that time, which was the first since 1975, a unique hiatus²². La Niña events are characterized by relatively cold surface temperatures in the equatorial Pacific and positive westerly wind anomalies at 200 mbar in the central, equatorial Pacific²³. Such conditions may be conducive to enhanced transport of trace gases between the hemispheres^{24,25}. Remembering that CH_4 mixing ratios are always larger in the Northern Hemisphere, any sudden, enhanced exchange of air between the hemispheres will lead to a temporary increase in the CH_4 growth rate in the Southern Hemisphere and a corresponding decrease in the Northern Hemisphere.

We describe the global budget of atmospheric CH_4 as a simple first-order differential equation

$$dM/dt = -kM + P \quad (2)$$

where M is the total atmospheric burden (Tg CH_4), P is the annual source term ($\text{Tg CH}_4 \text{ yr}^{-1}$) and k is the first-order loss rate (yr^{-1}), equivalent to the inverse of the average atmospheric lifetime. Differentiation of equation (2) gives

$$d^2M/dt^2 = -k dM/dt - M dk/dt + dP/dt \quad (3)$$

The second-derivative term is estimated directly from the fit of equation (1) to the global data as $-0.6 \text{ p.p.b. yr}^{-2}$ (see Table 1; twice the global a_3 coefficient), or -1.7 Tg yr^{-2} if we use $M = 4,800 \text{ Tg}$ for a global average CH_4 of $1,650 \text{ p.p.b.}$ For k we use a value of $(1/11) \text{ yr}^{-1}$, based on the recent redetermination²⁶ of the rate coefficient for reaction between CH_4 and OH and a globally weighted average tropospheric $[\text{OH}] = 8.7 \times 10^5$

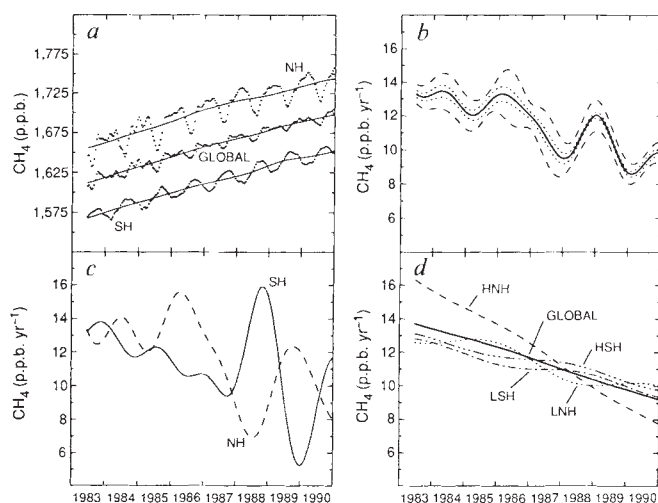


FIG. 3 *a*, Biweekly CH_4 mixing ratios (symbols) for global and Northern and Southern Hemisphere (NH and SH) regions calculated from the gridded values (Fig. 2), using cosine of latitude to weight for surface area. *b*, Growth-rate variations for the globally averaged CH_4 mixing ratios (solid line); uncertainties determined from a jack-knife method (dotted curves) and bootstrap method (dashed curves); see text. *c*, Growth-rate variation for the average methane mixing ratio in the Southern Hemisphere (solid line) and Northern Hemisphere (dashed line). *d*, Growth-rate variations for the global average methane mixing ratio and the four semi-hemispheres. In this panel, the results are shown for the case in which residuals have been filtered with a low-pass filter having a FWHM of $\sim 1,000$ days, instead of 1 year; the different filter suppresses interannual variations in the growth rate, and it highlights the result for the high Northern Hemisphere latitude zone.

molecule cm^{-3} (ref. 25). The average value of dM/dt is determined directly from our data as 34 Tg yr^{-1} , giving a value of 3.1 Tg yr^{-2} for $k dM/dt$.

The sum of the last two terms on the right side of equation (3) is therefore $+1.4 \text{ Tg yr}^{-2}$. If there has been no change in the first-order loss rate over the period of our measurements (that is, globally weighted OH has been constant, and the soil sink has not changed), the CH_4 source strength has been increasing at 1.4 Tg yr^{-2} ($0.3\% \text{ yr}^{-1}$ for $P = 470 \text{ Tg yr}^{-1}$), in good agreement with the value calculated recently using a similar approach²⁵. If alternatively the global sources are at steady state, then the global sink is decreasing by 1.4 Tg yr^{-2} ($0.3\% \text{ yr}^{-1}$). But such a decrease in the CH_4 sink is not in accord with calculations²⁵ suggesting that globally weighted OH may have grown at $1\% \text{ yr}^{-1}$ between 1978 and 1990. If we assume OH is increasing at this rate, our measurements imply that the global CH_4 source must be growing at 6 Tg yr^{-2} ($1.3\% \text{ yr}^{-1}$), also in agreement with that calculated using a similar approach²⁵. The time variation of the growth rate in each of the four semi-hemispheres is shown in Fig. 3*d*. The residuals have been passed through a filter with a FWHM of $\sim 1,000$ days, thus smoothing the growth-rate variations more than the filter with a FWHM of about 1 year. The most striking result is that the deceleration of the high Northern Hemisphere growth rate has been 2–3 times more rapid than in the other semi-hemispheres. This is supported by the values of the a_3 coefficients shown in Table 1. The lowest average CH_4 growth rate we observe is for the zone from the Equator to 30° S , and the next lowest is for the zone Equator– 30° N (Table 1). This is consistent with the hypothesis²⁵ that the increase in global OH is dominated by OH increases in the tropical lower troposphere. The CH_4 sources in the zone 30 – 90° N are both anthropogenic and natural¹⁴. Although it is not possible to determine from this study which particular sources have slowed their increase, the rapidity of the deceleration suggests that direct human actions may be responsible. The component of the global methane source that is most amenable to rapid reductions by

human intervention is that associated with fossil-fuel exploitation. □

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Antarctic ice volume and contribution to sea-level fall at 20,000 yr BP from raised beaches

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THE contribution of the Antarctic ice sheets to global sea-level fall at the Last Glacial Maximum (LGM) depends largely on how the extent and thickness of peripheral ice changed. Model studies^{1–3} suggest that there was widespread thickening (from 500 m to more than 1,000 m) of the ice-sheet margins, sufficient to induce a drop in sea level of at least 25 m. Geological evidence^{4,5}, on the other hand, indicates only limited ice expansion and a sea-level fall of just 8 m. Here we use recent data on the altitudes and ages of raised beaches from the Ross embayment and East Antarctica to investigate the timing and extent of Antarctic deglaciation. These indicate that the ice margin during the LGM was thinner and less extensive than has been formerly thought, and that its contribution to the drop in sea level was only 0.5–2.5 m. Deglaciation was well advanced by 10 kyr BP and was complete by 6 kyr BP. These findings imply either that sea level during the LGM fell less than present estimates suggest, or that an ice volume considerably greater than currently accepted must have been present in the Northern Hemisphere.

The Ross Embayment coast (Fig. 1)^{6,7} shows a northern group

TABLE 1 Holocene raised beach altitudes and radiocarbon dates

Ross Sea			
Cape Adare	4.5 m	$>2,680 \pm 150$	NZ 6096; corrected for reservoir effect (CRE) 1,090 yr
Adelie Cove	2.75 m	$>3,400 \pm 280$	NZ 6906; CRE 1,090 yr ³²
Inexpressible Island	21 m*	$>5,245 \pm 110$	GX 13615; CRE 1,090 yr ³³
Inexpressible Island	ice-free	$>7,505 \pm 230$	GX 14069; uncorrected
Franklin Island	10 m	$>3,690 \pm 80$	NZ 6357; CRE 1,090 yr
Cape Bird	ice-free	$>7,190 \pm 180$	NZ 5590; CRE 1,090 yr ³⁴
Marble Point	13 m*	$5,650 \pm 150$	L627; uncorrected ³⁵
East Antarctica			
Windmill Islands	31–32 m		
	23 m	$>6,040 \pm 250$	M1052; uncorrected ³⁶
Bunger Hills	8.5 m	$>7,650 \pm 490$	Beta 15828; CRE 1,300 yr ²⁷
Vestfold Hills	10 m	$>6,290 \pm 80$	SUA 2026; CRE 1,300 yr ^{21,37}
Soya Coast	16 m	$>10,250 \pm 210$	No laboratory number; uncorrected ¹²

* Heights of 21 m and 13 m probably 18.5 m and 9.5 m above the modern marine limit⁷.

of low altitude beaches focused on Cape Adare and a southern group of higher-altitude beaches exceeding 30 m between Inexpressible Island and Cape Ross. The isobase pattern shows uplift response to LGM ice centres in Victoria Land rather than the Transantarctic Mountains as required by the CLIMAP reconstruction for the Ross Sea^{3,8}.

The beaches are of middle to late Holocene age (Table 1) with the oldest at Cape Bird showing that Ross Island and McMurdo Sound were deglaciated by 7.2 kyr BP. Moraines at Adelie Cove, Inexpressible Island and Marble Point show LGM ice surfaces at 360 m, 550 m and 600 m (ref. 3).

Coastal areas in East Antarctica (Fig. 2) also show low-altitude raised beaches of middle and late Holocene age (Table 2). Radiocarbon dates show that the Windmill Islands (110° E) were deglaciated before 8160 ± 300 yr BP (ANU 6401)⁹; the Bunger Hills before $10,070 \pm 80$ yr BP (Lu 2032)¹⁰; the Vestfold Hills before $8,620 \pm 100$ yr BP (SUA 2924; Cre 1,300 yr)¹¹; and Soya Coast may have remained unglaciated during the LGM¹².

No Antarctic raised beach site allows construction of a sea-level curve. To assess LGM ice thickness and extent in the coastal zone, and Antarctica's contribution to post-glacial sea-level rise, we must consider glacial geological evidence from the coastal zone and external data for LGM global sea-level fall, and calculate isostatic uplift with its implications for ice thicknesses. Such determinations cannot be precise, but current data indicate thinner and less-extensive ice in coastal Antarctica, and a smaller contribution to sea-level fall than the CLIMAP maximum model³.

The LGM ice-sheet margin configuration usually adopted depends on extrapolation of outlet glacier profiles from the Transantarctic Mountains⁸ and models that assume that the continental shelf edge controlled the expansion of ice when sea level fell^{2,3}. Ice-core studies at Vostok¹³ and Dome C^{14,15} indicate that the surface of central Antarctica was 200–300 m lower at LGM than at present. In contrast, data from core D-10, 150 km inland of Dumont d'Urville, and from Law Dome suggest that ice in the coastal zone was ~1,300 m and 530 m thicker¹⁶. Drilling in Ross Sea¹⁷ and Prydz Bay¹⁸ has found no basal till of LGM age; most deposits are glaciomarine and of uncertain age. So apart from isotope data for core D-10, no data indicate ice thicker than 1,000 m in the coastal zone, and only the Mertz and Ninnis glaciers, which occupied deep troughs of Wilkes Land (140–150° E), extended to the shelf edge¹⁹.

Recent geomorphological studies in East Antarctica suggest that the ice-sheet margin at LGM was thinner and less extensive than previously thought. Raised beach studies at Windmill Islands (110° E) suggest an ice cover of 200–400 m grounded to the 200-m isobath 8–15 km offshore⁹. Similar studies at Bunger Hills (101° E) suggest ice 154–400 m thick and little extension beyond the Shackleton Ice Shelf²⁰. At Vestfold Hills (78° E) the ice was >158 m and probably <300 m thick²¹. Its northward limit is uncertain. Moraines 170 m above Lambert Glacier

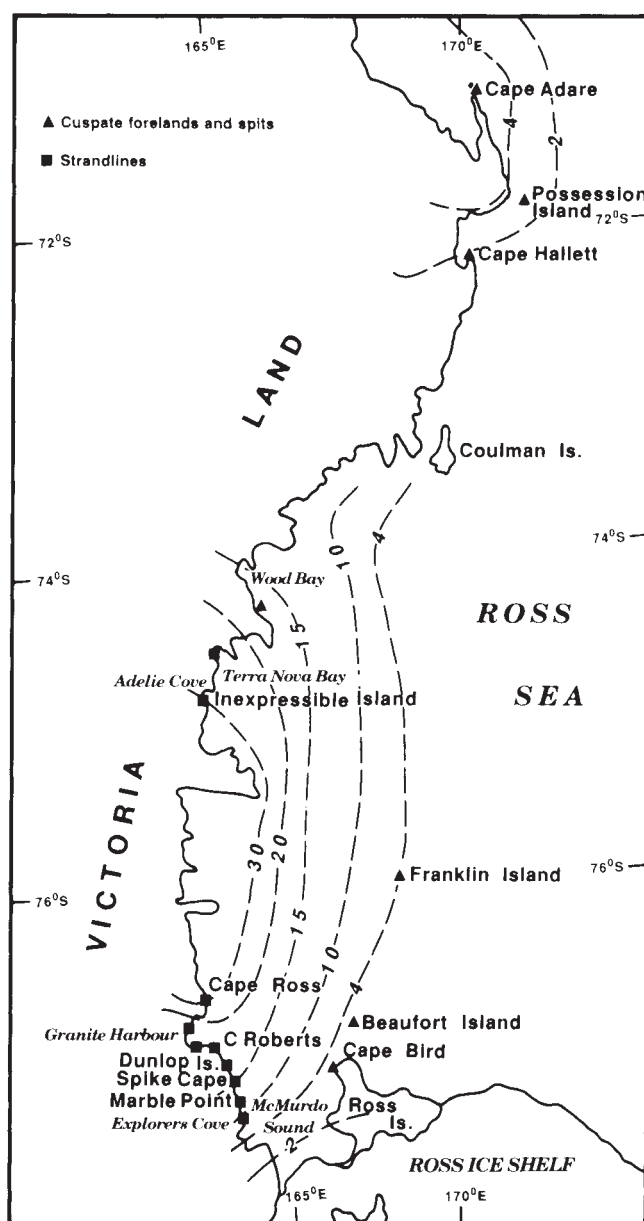


FIG. 1 Localities of raised beaches on the Victoria Land coast showing isobases defining two foci of uplift in northern and southern Victoria Land. After Kirk⁷.

TABLE 2 Estimates of LGM ice thicknesses in coastal Antarctica

	Maximum beach height (m)	Minimum age of deglaciation (kyr BP)	Residual uplift* (%)	Estimated ice thickness (m)	Ref.
Ross Embayment North	4.5	(2.7+) or 7.2+	6	383	22
				460	23
				17	24
South	32	7.2	6	484	22
				561	23
				124	24
Windmill Islands	32	8	5.4	484	22
				561	23
				124	24
Bunger Hills	8.5	10	0	398	22
				475	23
				31	24
Vestfold Hills	10	8.6	3.8	403	22
				480	23
				38	24
Soya Coast	16	10.2			
Uncorrected		~8.9	2.9	425	22
				502	23
				60	24

Ice thickness estimated: LGM sea level fall (100 m, ref. 22; 121 ref. 23) + raised beach height $\times$ mantle density 3.3 ($3,300 \text{ kg m}^{-3}$) $\times$ ice density 0.9 (900 kg m^{-3}). * See ref. 24.

(68° E) adjacent to Fisher Massif seem to mark the LGM. Such limited thickening of the lower Lambert Glacier is incompatible with ice extension across Prydz Bay to the shelf edge. As estimates of ice thickness and extent derived from onshore geomorphological evidence differ from the conclusions of glaciological models, it is important to examine what is implied by data from raised beaches.

Compared with Canada and Scandinavia, Antarctic raised beaches are very low. Because a sea-level curve cannot be obtained, the significance of the beach data must be based on inductive arguments. Clark and Lingle suggest that the sea level fell by 100 m at LGM, but because of land depression was only 1.5 m lower than at present in the Windmill Islands at 16 kyr BP²². The best estimate of world sea-level fall at the LGM is

121 ± 5 m (ref. 23). Andrews²⁴ has shown in Canada that by 10 kyr after deglaciation, uplift is complete. As Bunger Hills were deglaciated by 10 kyr BP and other coastal areas shortly afterwards, if not earlier, little residual uplift should remain in coastal Antarctica from LGM ice expansion. Uplift since LGM may therefore be estimated from values for sea-level fall, beach altitude, and ice and mantle density^{22,23}, or by the method of ref. 24 (Table 2). The effect of shelf flooding is omitted as there is little difference between LGM and present Antarctic sea levels²².

Ice thicknesses estimated from sea-level fall are near maximum values for two reasons. First, depression of coastal Antarctica considerably in excess of sea-level fall would give much higher beaches. Ice 1,000 m thick and a sea-level fall of

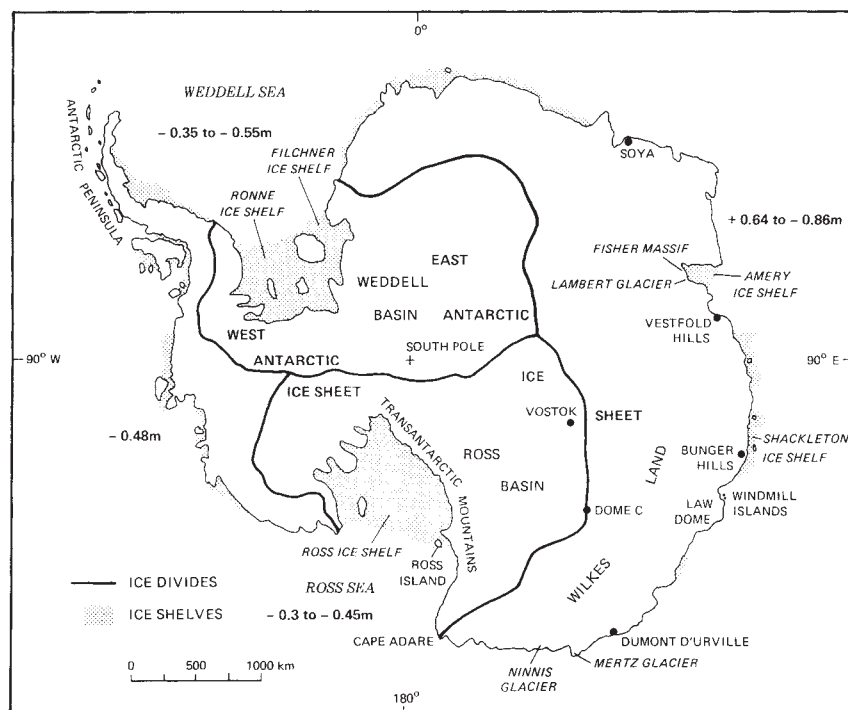


FIG. 2 Collective glacier catchments³¹ contributing to the East Antarctic Ice Sheet, the Ross Sea Basin, the Antarctic Peninsula and western Antarctic mountains. Calculation indicates that expansion of the EAIS contributed ~ 0.86 m to world sea-level fall, Ross Basin ice $0.3\text{--}0.45$ m, Weddell Basin ice $0.35\text{--}0.55$ m (by analogy with Ross), and the West Antarctic glacier systems 0.48 m giving a total contribution of $0.5\text{--}2.5$ m fall in world sea level at the Last Glacial Maximum.

121 m would produce beaches at 216 m altitude if fully uplifted ($1,000 \times (\text{ice density}) (\text{mantle density}) = 337 \text{ m} - 121 \text{ m} = 216 \text{ m}$). Second, the values generally exceed the geological evidence for increased ice thickness.

The radiocarbon dates used in Andrews' method are minimum values which provide maximum values for the residual uplift. The estimated ice thicknesses are clearly too low as the geological record indicates thicker ice on the inner shelf. This suggests a longer response time in Antarctica than in Canada for isostatic equilibrium to be established after deglaciation.

Although the extent and thickness of LGM ice on the continental shelf of Antarctica cannot yet be fully assessed most geological evidence indicates that it is unlikely to have reached the shelf edge throughout its periphery. An average thickness of 500 m and a grounding line advance of 30 km over the inner shelf seem to be reasonable estimates.

If we use these figures for ice increase in the coastal zone, together with an inland decrease in thickness to 0 m at the 2,000-m contour and a sea level fall of 120 m, we can estimate LGM increase in Antarctic ice volume and its probable contribution to sea-level fall. Integrated around the 9,100-km margin of East Antarctica between 170°E and 30°W (excluding Lambert Glacier), this accounts for a sea-level fall of 0.73 m (calculated as $32 \text{ km}^2 \times 9,100 \text{ km} \times 0.9 (900 \text{ kg m}^{-1})$ divided by the world sea-level area in km^2). The Lambert Glacier system contributed a fall of 0.13 m (assuming that the drainage area of $2.5 \times 10^5 \text{ km}^2$ below 2,000 m increased by 200 m) to give a total fall of 0.86 m due to LGM expansion of the Southern Ocean margin of the East Antarctic ice sheet. But ice-core studies^{13,15} indicate that the ice-sheet interior was 200–300 m lower. An average lowering of 150 m over the $4 \times 10^6 \text{ km}^2$ area of ice sheet above 2,000 m would add 1.5 m to sea level. Thus the net effect could have raised the sea level by 0.64 m. The full contribution of Antarctica to LGM sea level fall can be estimated similarly. Reconstruction for Ross Embayment⁸ indicates a contribution of $\sim 0.3\text{--}0.45 \text{ m}$ lowering. The slightly larger Weddell Embayment contributed $\sim 0.35\text{--}0.55 \text{ m}$. The remainder of the continent, including marginal West Antarctica and the Antarctic Peninsula, contributed 0.48 m. Our estimate for Antarctica's contribution to LGM sea-level fall is thus 0.5–2.5 m.

This is less than the 25 m of the CLIMAP and other models^{3,25,26}. As the figure of 120 m for LGM sea-level fall seems well constrained²³, our interpretation does not support these models and suggests either that the estimated fall of 120 m needs reconsideration, or that Northern Hemisphere ice sheets had greater volumes than previously calculated.

These data also allow the deglacial history of Antarctica to be broadly constrained. The East Antarctic ice-sheet margin had attained its present configuration by at least 8.5–9.0 kyr BP, and probably before 10 kyr BP^{12,23}. In Ross Embayment, the western margin of the Ross ice shelf was within 25 km of its present position by 7.2 kyr BP. In McMurdo Sound, the present coast was established before 6.6 kyr BP²⁸, and outlet glaciers on the Transantarctic Mountains were at their current elevations by 6 kyr BP²⁹.

Inputs to current models of sea-level change include data on the location, volume and melting histories of the LGM ice sheets^{25,26}. These models use the CLIMAP reconstruction of the Antarctic ice sheets to derive contributions to post-glacial sea-level rise of 25 m between 9 and 4 kyr BP²⁵ and 37 m between 18 and 6 kyr BP²⁶. Such estimates conflict with the geological data, suggesting that the models must be re-examined. This is important as the models are widely used to assess present patterns of sea-level change^{29,30}. □

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Correlation between isotope records in marine and continental carbon reservoirs near the Palaeocene/Eocene boundary

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CHANGES in the isotope content of the large marine carbon reservoir can force shifts in that of the smaller carbon pools in the atmosphere and on land. The carbon isotope compositions of marine carbonate sediments from the late Palaeocene vary considerably, exhibiting a sudden decrease close to the Palaeocene/Eocene boundary which coincides with deep-sea benthic extinctions¹ and with changes in ocean circulation. Here we report that these fluctuations in the marine carbon isotope record are closely tracked by the terrestrial records provided by palaeosol carbonates and mammalian tooth enamel. In using palaeosol carbonates to reconstruct the CO_2 content of the ancient atmosphere², isotope shifts of this sort will have to be taken into account. The sharp decrease in $^{13}\text{C}/^{12}\text{C}$ ratios in the late Palaeocene provides a datum for precise correlation of marine and continental records, and suggests that abrupt climate warming at this time may have played an important role in the evolution of land mammals.

From the late Palaeocene (~ 60 million years ago [Myr]) to the early Eocene (~ 55 Myr)³, the carbon isotope composition of biogenic marine carbonate decreased by 3‰ (ref. 4; Fig. 1). Superimposed on this trend is a short-term drop of 2.5–4.5‰, recorded in planktonic and benthic foraminifera from the latest Palaeocene (~ 57.3 Myr)¹. This unusual drop coincided with marine warming, a decrease in the surface-to-bottom water thermal gradient and mass extinction of benthic foraminifera^{5,6}.

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Currently this short-term carbon excursion has only been documented at Ocean Drilling Project (ODP) site 690, but it has been reported in the equatorial Pacific⁷, northern Atlantic⁷, southern Atlantic⁸ and Indian oceans^{8,9} and was most probably a global marine event. Because most carbon in the Earth's biosphere, atmosphere and hydrosphere is in the oceans¹⁰, the global fluctuations in marine isotopes in the Palaeocene/Eocene should have affected atmospheric and continental carbon reservoirs. Coupling of isotope signals between Permian marine carbonates and land vertebrate hydroxyapatite has been detected previously¹¹. We explore isotope coupling between marine carbonate and continental tooth apatite and palaeosol carbonate across the Palaeocene/Eocene boundary.

The isotope relationships between marine, atmospheric and continental carbon reservoirs are presented in Fig. 2. Atmospheric CO₂ is in long-term carbon isotope equilibrium with

TABLE 1 Measured $\delta^{13}\text{C}$ for each sampling horizon in Fort Union and Willwood formations

Level	Age (Myr)	$\delta^{13}\text{C}$ (‰) EA	$\delta^{13}\text{C}$ (‰) PC	Δ (‰) PC-EA
Eocene				
2,210	55.90	-12.2	-10.1	2.1
2,110	56.10	-12.0	-8.6	3.4
2,085	56.15	-12.9		
2,050	56.20	-11.6		
2,020	56.30	-12.2		
1,995	56.35	-11.1		
1,970	56.40	-12.4	-9.0	3.4
1,935	56.45	-11.4		
1,910	56.50	-12.3	-7.9	4.4
1,870	56.60	-12.2		
1,840	56.65	-11.4	-7.4	4.0
1,760	56.80	-12.0	-8.2	3.8
1,700	56.95	-11.5	-9.1	2.4
Palaeocene				
1,665	57.00	-12.4		
1,630	57.10	-11.4	-8.2	3.2
1,570	57.20	-11.3	-8.5	2.8
1,530	57.30	-13.9		
1,525	57.30	-12.3	-9.9	2.4
1,520	57.30	-15.7	-14.5	1.2
1,515	57.30	-12.0		
1,505	57.35	-9.8		
1,495	57.35	-9.7	-11.4	-1.7
1,460	57.45	-10.9		
1,425	57.55	-11.8		
1,385	57.70	-10.4		
1,355	57.75	-11.0	-8.0	3.0
1,330	57.85	-11.2		
1,280	58.00	-9.5	-6.6	2.9
1,250	58.05	-13.2		
1,210	58.20	-9.1	-8.5	0.6
1,150	58.35	-11.2		
1,110	58.45	-11.0		
1,080	58.55	-10.1	-7.4	2.7
1,050	58.65	-9.0	-8.2	0.8
1,015	58.75	-10.7	-7.0	3.7
940	58.95	-9.9	-8.3	1.6
860	59.15		-7.0	
760	59.45	-7.4	-6.6	0.8
720	59.55	-11.0		
700	59.60	-8.7	-7.6	1.1
680	59.65	-10.5		
550	60.00	-9.2		

EA, herbivore tooth enamel apatite; PC, palaeosol carbonate; difference between these materials, $\Delta_{\text{PC-EA}} = \delta^{13}\text{C}_{\text{PC}} - \delta^{13}\text{C}_{\text{EA}}$. Level refers to metres above K/T boundary on Polecat Bench, Bighorn Basin, Wyoming. Ages calculated at 50,000-year intervals by linear interpolation using dates from Fig. 3.

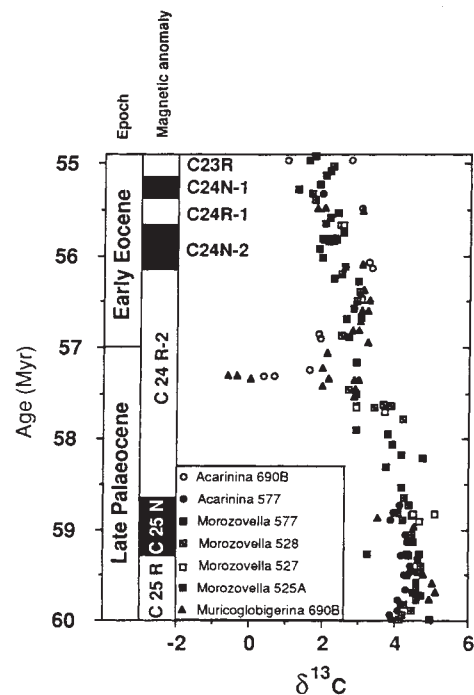


FIG. 1 Fluctuations in $\delta^{13}\text{C}$ of near-surface planktonic foraminifera from marine waters across the Palaeocene/Eocene boundary. Note the gradual decrease from 60 Myr to 55 Myr, and the brief (<50,000 year) negative excursion at 57.3 Myr. The data are compiled from ODP holes 690B (Weddell Sea, Antarctica)³⁰, 525A, 527 and 528 (Walvis Ridge, south Atlantic)³¹, and 577 (Shatsky Rise, south Pacific)³². Absolute ages follow Aubry *et al.*³, but age assignments will be shifted ~2 Myr younger by impending revisions of Palaeogene dating and stratigraphy (C. Swisher, personal communication)²⁷. $\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C})_{\text{sample}}/({}^{13}\text{C}/{}^{12}\text{C})_{\text{standard}} - 1]$, where standard is PDB.

calcium carbonate in the tests of surficial marine organisms, with a temperature-dependent offset of ~9‰ (ref. 12). These carbon reservoirs may decouple transiently, but equilibration should occur rapidly because of the high rates of marine carbon turnover (<5,000 years) and exchange between the ocean mixed layer and the atmosphere (<50 years)¹⁰.

As land plants fix atmospheric CO₂ by photosynthesis, they fractionate carbon isotopes, preferentially incorporating ¹²C. Although different photosynthetic pathways (C3, C4 or CAM) exist today, Palaeocene/Eocene floras were dominated by C3 plants^{13,14}. Modern C3 plants and atmospheric CO₂ differ by -19.5 ± 2‰ (ref. 15). Vertebrate herbivores eat plants, and deposit carbonate hydroxyapatite in their teeth that differs in ¹³C content ($\delta^{13}\text{C}$; see Fig. 1) from their diets by +12 ± 1‰ (ref. 16). Thus land plants and herbivores track changes in the $\delta^{13}\text{C}$ of atmospheric CO₂ that are ultimately driven by the ocean.

The CO₂ gas deep in a soil is supplied by decay of plants and root respiration, which produce CO₂ that is isotopically similar to source plants¹⁷. Atmospheric CO₂, which has a less negative $\delta^{13}\text{C}$ than plants, mixes into soils, impeding the outward diffusion of soil CO₂. Consequently, the $\delta^{13}\text{C}$ of soil CO₂ is higher than purely plant-derived CO₂ (ref. 18). Below ~30 cm, soil CO₂ is isotopically homogeneous, and soil carbonates from these depths, which form in isotopic equilibrium with soil CO₂, have $\delta^{13}\text{C}$ values ~15‰ greater than the overlying flora^{17,19}. Model experiments have shown, however, that if the atmospheric concentration of CO₂ (p_{CO_2}) was higher in the past, atmospheric penetration into soils would be greater, increasing both the $\delta^{13}\text{C}$ of soil carbonates and the difference in $\delta^{13}\text{C}$ between carbonate and plants². These model results have been developed into a method for estimating p_{CO_2} using the $\delta^{13}\text{C}$ of palaeosol carbonate^{2,20}.

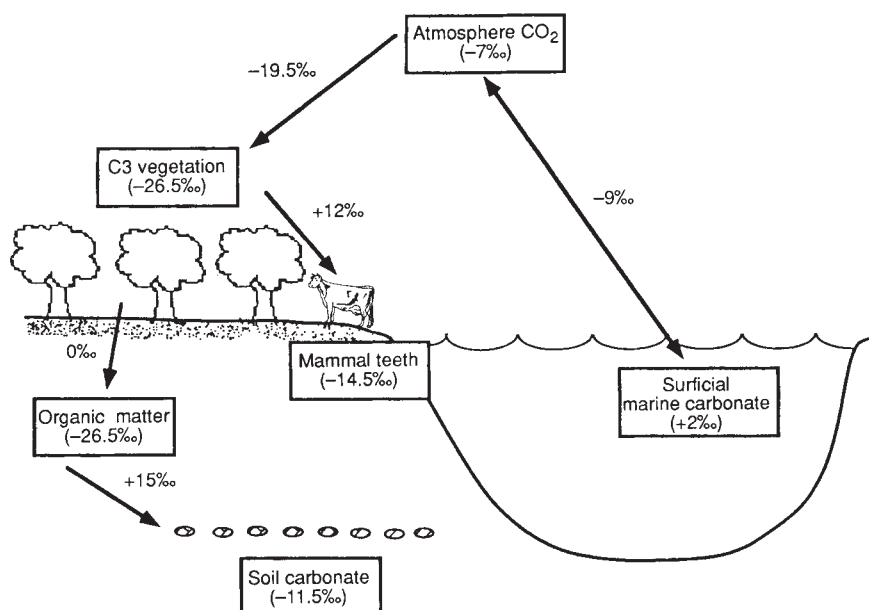


FIG. 2 Model of coupling between marine, atmospheric and continental carbon reservoirs. Equilibrium fractionation between surficial marine carbonate and atmospheric CO_2 is $\sim -9\%$ when temperatures range from 20–30 °C (ref. 12). The model assumes that Palaeocene/Eocene floras had only C3 plants; the fractionation characteristic of these plants (-19.5%) was used¹⁵. The modern value for the difference between plants and soil carbonate was used (15%), but this value would increase if atmospheric p_{CO_2} was greater than its present value². Palaeosol carbonate $\delta^{13}\text{C}$ are $\sim 3\%$ greater than enamel apatite $\delta^{13}\text{C}$, but this difference would also increase if p_{CO_2} were higher. Typical values for modern C3 ecosystems are supplied in boxes.

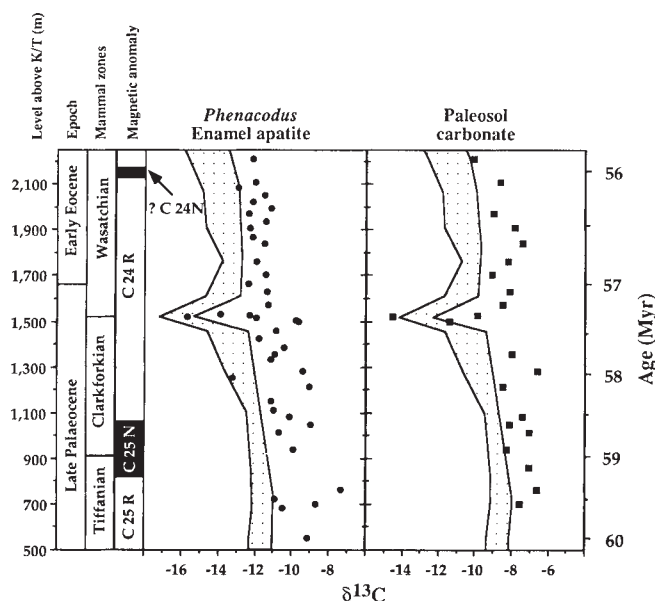


FIG. 3 Trends in $\delta^{13}\text{C}$ across the Palaeocene/Eocene boundary in the Bighorn Basin, Wyoming: points are from enamel apatite and palaeosol carbonate, shaded regions are values predicted from biogenic marine carbonate. Predicted values were calculated at 500,000-year intervals using the high and low $\delta^{13}\text{C}$ values for marine carbonates from Fig. 1. We first calculated a range of $\delta^{13}\text{C}$ values for atmospheric CO_2 , assuming equilibrium fractionation with high and low $\delta^{13}\text{C}$ values for marine carbonate at 20 and 30 °C. We then applied values from Fig. 2 for fractionations between atmosphere, plants, enamel apatite and palaeosol carbonate, and plotted the maximum calculated range for each interval. Level refers to metres above the K/T boundary in the Clarks Fork region^{21,22}. Marine and continental correlation and ages were determined using four points, including the bottom of C25R at 60.2 Myr, the bottom of C24R-2 at 58.6, and the age of 57.3 for carbon isotope excursions. The fourth point was an age of 55.9 Myr for the base of Wa5, which was correlated using mammalian biostratigraphy to the southern Bighorn Basin. An age for this level in the southern Bighorn Basin was obtained by linear interpolation between an ^{40}Ar – ^{39}Ar dated level in the upper Wasatchian, and a date for a level in the Clarkforkian supplied by palynofloral correlation to the marine NP9/NP10 boundary²⁷.

We sought evidence of isotope coupling between the oceans and continents by analysing mammal teeth and palaeosol carbonates from the continental Fort Union and Willwood formations in the Bighorn Basin, northwestern Wyoming, which provide a continuous sequence spanning the Palaeocene/Eocene boundary (Fig. 3)^{21,22}. Tooth enamel was collected from the herbivorous mammal, *Phenacodus*, then treated with NaOCl and dilute acetic acid to remove organic matter and diagenetic carbonates²³. Unlike bone apatite, which is highly susceptible to post-mortem alteration, enamel apatite is resistant, owing to its greater crystal size and lower porosity^{23,24}. Palaeosols occurred as thin, organic-rich A horizons (when present) overlying thicker yellow, red, orange or purple B horizons²⁵. We collected micritic carbonate nodules from ≥ 30 cm below original palaeosol surfaces, and isotope samples from nonrecrystallized zones in nodules. Samples were analysed on a Finnigan MAT 251 mass spectrometer linked to a Kiel automatic carbonate preparation device. Carbonate and apatite were reacted at 75 °C for 5 and 15 minutes, respectively. Samples were calibrated to PDB using NBS 20, with a precision of $\leq 0.1\%$.

The gradual decrease in marine $\delta^{13}\text{C}$ from late Palaeocene

(60 Myr) to early Eocene (56 Myr) was closely tracked by enamel apatite and palaeosol carbonate. The $\delta^{13}\text{C}$ of palaeosol carbonate decreased from -7 to -10% , whereas enamel apatite dropped from -8 to -12% (Table 1, Fig. 3). Peak values for land and marine $\delta^{13}\text{C}$ occurred within C25R, and values for both records decreased through C25N and C24R (Figs 1 and 3).

Continental materials also record an extraordinary drop precisely at the Clarkforkian/Wasatchian (Cf/Wa) boundary (Fig. 3). The $\delta^{13}\text{C}$ of enamel apatite and palaeosol carbonate dropped by 5.9 and 4.5%, respectively, in less than 50,000 years; these low values persisted for less than 100,000 years (Fig. 3). This continental carbon isotope excursion was very similar to the latest Palaeocene marine carbon excursion in magnitude, direction and duration (Figs 1 and 3, ref. 1). When the marine and continental records are correlated, using magnetostratigraphy^{3,21}, palynoflora and radiometric dates^{26,27} (Fig. 3), estimates for the age of the marine and continental excursions differ by only 150,000 years. This apparent age difference is remarkably small, particularly when compared with the uncertainties for the ages of the isochrons used to correlate continental and marine strata. Given their strong similarities in timing and

character, we conclude that the latest Palaeocene marine excursion in isotope ratios and the continental excursion at the Cf/Wa boundary were isochronous.

Differences in $\delta^{13}\text{C}$ between palaeosol carbonate and enamel apatite were similar to differences between these components today (Table 1). Furthermore, palaeosol carbonate and enamel apatite had $\delta^{13}\text{C}$ values close to those predicted from marine carbonate (Fig. 3). The persistent 1–2‰ offset between predicted and observed values is puzzling. A diagenetic explanation is unlikely, because it would entail identical alteration of two different minerals, apatite and calcite, to maintain the appropriate isotope spacing observed between the minerals. A small fraction of C4 plants in the Palaeocene/Eocene could explain the offset. This idea is not supported by most palaeobotanical or isotope evidence from fossil organic matter^{13,14}, although the possibility of non-C3 plants before the Miocene is controversial²⁸. We attribute the offset to uncertainties in values for model parameters, particularly for ocean-atmosphere and atmosphere-plant fractionations. Atmosphere-plant fractionation, for example, covaries with temperature, light intensity and $p\text{CO}_2$ (ref. 15), all of which may have been different in the Palaeocene/Eocene. Given these possible sources of error, the model and observed records are remarkably similar.

Coupling of marine and continental carbon reservoirs has three important implications. To determine ancient $p\text{CO}_2$ using palaeosol carbonates, the $\delta^{13}\text{C}$ of ancient C3 plants and atmospheric CO_2 must be estimated. In past applications of the method, it was argued that these carbon reservoirs were isotopically constant through geological time^{2,20}. We have demonstrated, however, that the $\delta^{13}\text{C}$ of C3 plants and atmospheric CO_2 can vary substantially; thus they must be independently monitored. We used Cerling's model² to recalculate Palaeocene/Eocene atmospheric $p\text{CO}_2$, but estimated the $\delta^{13}\text{C}$ of C3 plants and atmospheric CO_2 using enamel apatite. Throughout the entire interval, including the excursion at the Cf/Wa boundary, we calculated $p\text{CO}_2$ values of ≤ 350 p.p.m.v., lower than Cerling's result (< 750 p.p.m.v.), calculated assuming modern $\delta^{13}\text{C}$ for C3 plants and atmospheric CO_2 . Yet both studies indicated relatively low $p\text{CO}_2$ values (1–2 times modern levels) at this time, consistent with independent estimates from modelling experiments²⁹.

The sharp, isochronous, global drop in $\delta^{13}\text{C}$ provides a datum linking the marine and continental realms near the Palaeocene/Eocene boundary, an interval of considerable stratigraphic controversy^{3,21,26,27}. The marine isotope excursion and the benthic extinction immediately precede the marine Palaeocene/Eocene boundary^{1,3,4}, which lies within calcareous nannoplankton zone NP10 and within planktonic foraminiferal zone P6b. On land, the isotope excursion marks the Cf/Wa boundary. Thus the currently defined marine Palaeocene/Eocene boundary corresponds to a level within the earliest Wasatchian land mammal age.

The carbon isotope excursion links the sudden extinction of deep sea organisms with the onset of evolutionary changes in late Palaeocene land mammals, including the first appearance of important groups such as artiodactyls, perissodactyls and primates²². The benthic extinction has been attributed to sudden change in ocean circulation, possibly resulting in deep-sea warming^{1,6}. Perhaps abrupt climate warming spurred morphological changes in mammals, or perhaps the brief period ($\sim 100,000$ years) of warmth in high latitudes permitted dispersal of new forms between continents. The marine-continental linkage provided by carbon isotopes indicates a more prominent role for climate warming in the evolution of Palaeogene land mammals than was previously realized. □

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Stability of high-density clinoenstatite at upper-mantle pressures

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SILICATE pyroxenes are major components in mineralogical models of the Earth's upper mantle^{1,2}, with the transformation of chain-silicate pyroxenes to denser garnet structures being a possible cause³ of the seismic discontinuity at 400 km depth that divides the upper mantle from the transition zone. At shallower depths assemblages containing two pyroxenes are stable: calcium and sodium components are accommodated in a diopside-jadeite solid solution³, while magnesium and iron form a second, calcium-poor pyroxene. In the absence of experimental data, orthoenstatite was long believed to be the stable polymorph of (Mg, Fe)-pyroxene over the entire upper mantle. More recently, however, petrological experiments^{4,5} at pressures and temperatures in excess of 8 GPa and 900 °C have provided evidence for the transformation of Mg-orthopyroxene to a clinopyroxene phase. The thermodynamic and physical properties of this phase are completely unknown. Here we report the results of a high-pressure single-crystal diffraction study which confirm the stability of a high-clinopyroxene phase of MgSiO_3 at high pressures, and allow an initial estimate to be made of the density changes associated with the transformation of the orthopyroxene component in the Earth's upper mantle.

All of the structures of the pyroxene polymorphs of MgSiO_3 are based on single chains of corner-sharing tetrahedra which cross-link, by sharing oxygen atoms, parallel bands of octahedrally coordinated magnesium atoms (Fig. 1). The various polymorphs differ in the conformations of the silicate chains and in the relative positions of successive layers of octahedral strips. The stable phase of MgSiO_3 at ambient pressure and temperature is low clinoenstatite, with orthoenstatite becoming

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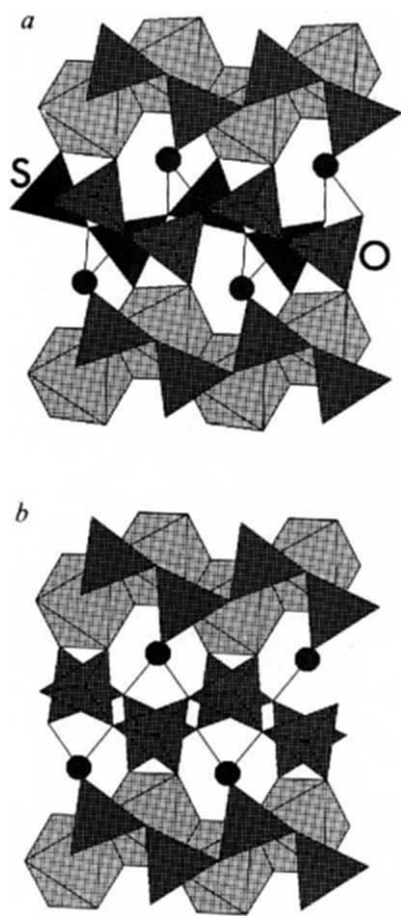


FIG. 1 Polyhedral representations of portions of the structures of (a) low clinoenstatite at ambient pressure⁷ and (b) high clinoenstatite at 7.93 GPa. SiO_4 groups are represented by tetrahedra, M1 sites by octahedra and M2 by circles. Note the change in the configuration of the lower chain, labelled 'S' in a, from low to high clinoenstatite. This change results in different arrangements of the bonds (drawn as lines) between the M2 sites and the bridging oxygens of the tetrahedral chains, and consequently smaller M2 sites in the high-clinoenstatite polymorph.

stable above $\sim 600^\circ\text{C}$. This phase boundary was experimentally reversed⁶ at 0.2 and 0.4 GPa, and has a slope of 0.022 GPa K^{-1} . Experimental studies^{4,5} at higher pressures and temperatures have reversed a second phase boundary with a much shallower pressure-temperature (P - T) slope⁴ of 0.0031 GPa K^{-1} between orthoenstatite as the low-pressure phase, and a low clinoenstatite as the quench product of a high-pressure phase. Two interpretations of this second phase boundary have been proposed. The first is that both phase boundaries represent the same transformation between orthoenstatite and low clinoenstatite, with Grover's experimental reversals⁶ being displaced by either shear stresses within his apparatus, or some other unexplained effect⁵. The second is that the high-pressure boundary represents a distinct transformation from orthoenstatite to a new polymorph that reverts to low clinoenstatite on quenching⁴. In the latter case we would expect the high-pressure phase to have thermodynamic and physical properties quite distinct from those of the well-known low clinopyroxene polymorph of MgSiO_3 . To distinguish between these two interpretations we have undertaken a high-pressure X-ray diffraction study of low clinoenstatite, to determine whether it is indeed formed as a quench product from high clinoenstatite.

Preliminary X-ray diffraction experiments on a single crystal of low clinoenstatite at ambient pressure gave identical unit-cell and structural parameters to those previously reported⁷. High-pressure experiments were done with this crystal in a

DXR-4 diamond-anvil pressure cell, with a 4:1 mixture of methanol:ethanol as the hydrostatic pressure-transmitting fluid. Pressures were obtained by the ruby fluorescence technique⁸. The unit-cell parameters, determined from the centred⁹ positions of 15–20 accessible low-angle X-ray diffraction maxima, vary smoothly with pressure up to 6.98 GPa. There was no significant variation in the intensities of the $h+k=2n+1$ reflections characteristic of low clinoenstatite. When we increased the pressure further to 7.93 GPa, the diffraction pattern changed discontinuously: the unit-cell parameters a and c decreased by $\sim 2\%$ and β changed from $107.74(2)^\circ$ at 6.98 GPa to $101.50(3)^\circ$. X-ray intensity data were collected from the crystal at this pressure (Table 1). The absence of all reflections with $h+k=2n+1$ from the diffraction pattern suggested that the structure had been transformed from low clinoenstatite to a pyroxene structure with $C2/c$ symmetry. The subsequent structure refinement, initiated with atomic coordinates of a computer simulation¹⁰ of high clinoenstatite at 10 GPa, converged rapidly to the final coordinates presented in Table 1, confirming the structure type as a C-centred monoclinic pyroxene.

The transformation from low to high clinoenstatite can be explained simply in terms of the geometrical constraints of the structure, and the coordination requirements of the cations. The two polymorphs of MgSiO_3 share the same stacking sequence of octahedral strips, but differ in the configuration of their silicate chains. In the structure of low clinoenstatite (Fig. 1a) there are two symmetrically distinct tetrahedral chains rotated in opposite directions (denoted¹¹ O and S), whereas in high clinoenstatite there is only one symmetrically distinct tetrahedral chain, which is O -rotated (Fig. 1b). The S configuration of chains allows for larger, but more distorted, sites for the magnesium cations occupying the M2 position than do the O -rotated chains. As SiO_4 tetrahedra are relatively incompressible, increasing pressure will tend to compress the MgO_6 octahedra differentially and the high-clinoenstatite structure, which contains only O -rotated chains and therefore small M2 sites, will become

TABLE 1 Crystallographic data for MgSiO_3 high clinoenstatite at 7.93 GPa

Positional and thermal parameters					
Site	Occupancy	x	y	z	B _{iso}
M1	Mg	0	0.9057(6)	1/4	0.34(7)
M2	Mg	0	0.2742(5)	1/4	0.57(8)
T	Si	0.2988(4)	0.0915(3)	0.2112(4)	0.29(4)
O1	O	0.1215(9)	0.0914(8)	0.139(1)	0.4(1)
O2	O	0.380(1)	0.2376(7)	0.367(1)	0.4(1)
O3	O	0.357(1)	0.0613(6)	0.915(1)	0.3(1)
Interatomic distances (Å)*					
M1-O1	2.087(8) [2]	M2-O1	2.067(8) [2]	T-O1	1.598(9) [1]
M1-O2	2.011(5) [2]	M2-O2	1.983(7) [2]	T-O2	1.582(7) [1]
M1-O2	1.975(8) [2]	M2-O3	2.195(8) [2]	T-O3	1.668(8) [1]
				T-O3	1.676(6) [1]
Average	2.024		2.082		1.631
Volume (Å ³)	10.95		11.90		2.21
QE†	1.007		1.011		1.005

Diffraction experiments with Krisel-automated Picker diffractometer, Mo $K\alpha$ radiation, $\lambda=0.7107\text{ \AA}$, scan mode ω , ambient temperature. $a=9.201(3)\text{ \AA}$, $b=8.621(1)\text{ \AA}$, $c=4.908(1)\text{ \AA}$, $\beta=101.50(3)^\circ$; $V_{\text{cell}}=381.5(2)\text{ \AA}^3$. Space group $C2/c$; $Z=8$, molar volume at 7.93 GPa= $28.72(2)\text{ cm}^3\text{ mol}^{-1}$. Data collected were 516 symmetry-allowed accessible reflections out to $\sin \theta/\lambda=0.7\text{ \AA}^{-1}$. Intensities were corrected for Lorentz and polarization effects and absorption by the components of the pressure cell. Data averaged in Laue group $2/m$ with $R_{\text{int}}=0.060$ for 239 symmetry-independent reflections, $R_{\text{int}}=0.026$ for 136 reflections with $I>3\sigma_I$ included in the structure refinement. Least-squares refinements were carried out with RFINE-88, a development version of RFINE4 (ref. 21). Complex scattering factors for neutral atoms were taken from International Tables²². Final refinement indices: $R_u=0.041$, $R_w=0.033$, $G_{\text{rit}}=1.74$.

* Figures in square brackets indicate bond multiplicities.

† QE is the quadratic elongation of each polyhedron²³.

energetically favoured. The actual mechanism of the phase transformation therefore involves a re-configuration of the silicate chains between 6.98 GPa and 7.93 GPa which brings the half of the tetrahedral chains that are *S*-rotated through a fully extended chain configuration and on into an *O*-rotated configuration.

On reduction of the pressure in the diamond-anvil cell, the high-clinoenstatite phase was retained down to 5.34 GPa, at which pressure diffraction maxima corresponding to low clinoenstatite reappeared and both polymorphs coexisted. Further pressure reduction resulted in the complete conversion of the sample back to low clinoenstatite. Our reversal of the transformation leads to two important conclusions. First, it demonstrates that high clinoenstatite has a true stability field at high pressures, and that the phase boundary reversed by the experimental studies^{4,5} at high pressures and temperatures is between orthoenstatite and high clinoenstatite. The tentative identification of a MgSiO_3 polymorph synthesized at 1,500 K at 16.5 GPa in a laser-heated diamond-anvil cell as high clinoenstatite (from Raman spectra collected *in situ* at high pressures)¹² is confirmed. Second, the phase transition between high and low clinoenstatite is nonquenchable, so that material equilibrated at high pressures and temperatures within the stability field of high clinoenstatite will revert to low clinoenstatite on quenching. Figure 2 shows the general topology of the phase diagram of MgSiO_3 that follows from our results and the experimental determinations of the transformation boundaries between low-clinoenstatite and orthoenstatite⁶, and between orthoenstatite and high clinoenstatite^{4,5}.

From six measurements between 7.93 and 5.34 GPa of the unit-cell volumes of high-clinoenstatite, and from the equation of state of low-clinoenstatite, we obtain an average volume change, $\Delta V = -0.80 \text{ cm}^3 \text{ mol}^{-1}$, for the low-clinoenstatite to high-clinoenstatite transformation. In the absence of good constraints for the equation of state of high clinoenstatite, we used that of low clinoenstatite to estimate the molar volume of high clinoenstatite at standard temperature and pressure (STP) to be $30.43 \text{ cm}^3 \text{ mol}^{-1}$, compared to $31.29(1) \text{ cm}^3 \text{ mol}^{-1}$ for low clinoenstatite. Because the difference in volumes at STP between low and high clinoenstatite ($0.86 \text{ cm}^3 \text{ mol}^{-1}$) is far larger than that between low clinoenstatite and orthoenstatite⁷ ($0.045 \text{ cm}^3 \text{ mol}^{-1}$) it will dominate ΔV_0 of the transformation of orthoenstatite to high clinoenstatite, which is calculated to be $-0.91 \text{ cm}^3 \text{ mol}^{-1}$. Associated estimates of ΔH_0 and ΔS_0 for this transition obtained from the experimentally determined phase boundary⁴ are 4.5 kJ mol^{-1} and $-2.8 \text{ J mol}^{-1} \text{ K}^{-1}$ respectively. Alternatively if one assumes that the ΔV of the low clinoenstatite to high clinoenstatite transition is independent of pressure, the thermodynamic parameters of the orthoenstatite to high-clinoenstatite transition become: $\Delta V_0 = -0.84 \text{ cm}^3 \text{ mol}^{-1}$, $\Delta H_0 = 4.2 \text{ kJ mol}^{-1}$, $\Delta S_0 = -2.6 \text{ J mol}^{-1} \text{ K}^{-1}$. These values are much larger than those obtained for the low clinoenstatite to orthoen-

statite transition, but are comparable to those of the orthopyroxene to high-clinopyroxene transition in MgGeO_3 (ref. 13). The value of ΔS_0 also agrees well with an estimate of $-2.8 \text{ J mol}^{-1} \text{ K}^{-1}$ obtained from Raman data¹², and the value of $-2.75 \text{ J mol}^{-1} \text{ K}^{-1}$ obtained¹⁴ from thermodynamic modelling of the enstatite-diopside join at low pressure. These values represent $\sim 10\%$ of the estimates of ΔH_0 , $\sim 30\%$ of ΔV_0 , and up to 100% of some estimates of ΔS_0 , for the high-pressure breakdown reactions of pyroxene to garnet or to Mg_2SiO_4 wadsleyite plus SiO_2 stishovite¹⁵. It is clear, therefore, that in calculations of phase equilibria between MgSiO_3 pyroxene and other phases at higher pressures the orthopyroxene inversion to high clinopyroxene cannot be ignored as previously suggested¹⁵.

Our results show that high clinopyroxene is an important mantle-forming phase with structure and thermodynamic properties quite distinct from the other polymorphs of MgSiO_3 . Because many of the principal seismic discontinuities in the Earth's mantle are attributed to isochemical phase changes in the dominant chemical components, it will be interesting to evaluate the effect that the orthoenstatite inversion to high clinoenstatite would have on seismic properties of the upper mantle. The Lehmann discontinuity, observed at depths between 180 km and 280 km in the subcontinental mantle¹⁶, but absent from globally stacked datasets¹⁷, has not been previously explained as an isochemical phase change because no experimental evidence existed for a plausible phase transition in a major mantle component. At the estimated pressure¹⁸ at 220 km depth, 7.1 GPa, the orthoenstatite to high clinoenstatite transition occurs at $\sim 700^\circ \text{C}$ in pure MgSiO_3 , far too cool a temperature for this depth in the subcontinental mantle. Synthesis experiments¹⁹ do however suggest that the same phase boundary in FeSiO_3 occurs at higher temperatures, whereas recent experimental data²⁰ suggest that the effects of Ca and Al are negligible. The density contrast associated with the orthoenstatite to high clinoenstatite phase transition is $\sim 3\%$. Birch law systematics suggest that the associated change in compressional velocity, v_p , would be 3–4%. If this pyroxene component formed as much as 20–25% of the upper mantle in the 180–280 km depth interval, the associated velocity increase would be 0.5–1.0%, compared with the 1.1–2.0% increase in v_p at the Lehmann discontinuity estimated¹⁶ from seismic observations. Clearly more experimental data are required, but it is nevertheless tempting to speculate that an orthoenstatite to high-clinoenstatite transition in (Mg, Fe) pyroxene may well contribute to this discontinuity. □

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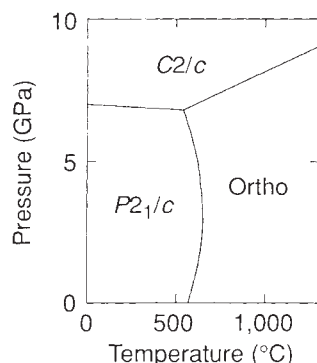


FIG. 2 General topology of the phase diagram of MgSiO_3 . The phase boundary between orthoenstatite and high clinoenstatite is taken from ref. 4, that between low clinoenstatite and orthoenstatite up to 0.4 GPa from ref. 6.

Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin

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INTRODUCTION of Ca^{2+} indicators (photoproteins, fluorescent dyes) that can be trapped in the cytosolic compartment of living cells has yielded major advances in our knowledge of Ca^{2+} homeostasis^{1,2}. Ca^{2+} however regulates functions not only in the cytosol but also within various organelles^{3,4} where indicators have not yet been specifically targeted. Here we present a novel procedure by which the free Ca^{2+} concentration of mitochondria, $[\text{Ca}^{2+}]_m$, can be monitored continuously at rest and during stimulation. The complementary DNA for the Ca^{2+} sensitive photoprotein aequorin was fused in frame with that encoding a mitochondrial presequence. The hybrid cDNA was transfected into bovine endothelial cells and stable clones were obtained expressing variable amounts of mitochondrially targeted apoaequorin. The functional photoprotein could be reconstituted in intact cells by incubation with purified coelenterazine and $[\text{Ca}^{2+}]_m$ could thus be monitored *in situ*. This allowed the unprecedented direct demonstration that agonist-stimulated elevations of cytosolic free Ca^{2+} , $[\text{Ca}^{2+}]_i$, (measured in parallel with Fura-2) evoke rapid and transient increases of $[\text{Ca}^{2+}]_m$, which can be prevented by pretreatment with a mitochondrial uncoupler. The possibility of targeting aequorin to cellular organelles not only offers a new and powerful method for studying aspects of Ca^{2+} homeostasis that up to now could not be directly approached, but might also be used in the future as a tool to report *in situ* a variety of apparently unrelated phenomena of wide biological interest.

The coelenterate photoprotein aequorin, composed of an apoprotein with a relative molecular mass of 22,000 (M_r 22K) and a hydrophobic prosthetic group (coelenterazine), emits light upon calcium binding⁵ and is widely used for measuring $[\text{Ca}^{2+}]_i$. Recently, endogenous production in the cytoplasm has been achieved by transfecting cells within the aequorin cDNA⁶⁻⁸. Here we demonstrate that functional aequorin can be targeted to an intracellular organelle, the mitochondrion, allowing the measurement of its matrix $[\text{Ca}^{2+}]$ in intact cells.

It has been already demonstrated that large protein domains can be fused to the N terminus of aequorin, without affecting its response to $[\text{Ca}^{2+}]$ (ref. 9). Thus a cDNA fragment encoding

the targeting presequence of subunit VIII of human cytochrome c oxidase¹⁰ was fused in frame with a portion of the aequorin cDNA encoding all the amino acids, but the first, of the mature polypeptide¹¹. The construction strategy of the chimaeric cDNA is summarized in Fig. 1. The cDNA (mtAEQ) was inserted in the cloning site of pMT2¹² and cotransfected into bovine endothelial cells with the pSV2-neo plasmid¹³. After selection, six independent G418-resistant clones were isolated, expanded and analysed for integration of mtAEQ DNA in the genome and for functional aequorin expression (Fig. 2). A quantitative polymerase chain reaction (PCR) shows that all clones have mtAEQ sequences integrated in the genome in different amounts, but no correlation was observed with the largely variable expression of functional aequorin. Similar results (1 highly expressing clone out of 4) were obtained when PC12 clones were isolated after transfection with the plasmid pRSVAQ¹⁴, which induces expression of cytosolic aequorin (R.R. *et al.*, unpublished results). All the following analyses were thus performed with the clone (M4) producing the highest amount of recombinant aequorin (roughly 3×10^4 molecules per cell).

Evidence was sought of the intramitochondrial localization of the photoprotein. Table 1 shows that in the clone M4, digitonin permeabilization of the plasma membrane¹⁵ caused almost complete release of the cytosolic enzyme lactate dehydrogenase. In contrast, the mitochondrial enzyme glutamate dehydrogenase and recombinant aequorin could be recovered in the soluble supernatant only after freeze-thawing of the pellet, as expected for proteins segregated within membrane-bound structures. The same results were obtained with a different clone (M1); in all clones, no aequorin activity was present in the freeze-thaw pellet (not shown). That aequorin was localized within mitochondria was more directly proven by the functional evidence presented in Fig. 3.

Functional aequorin was reconstituted *in situ* by incubating overnight intact M4 cells in the presence of coelenterazine. In resting, unstimulated cells, the number of photons emitted was close to background. The effect on $[\text{Ca}^{2+}]_m$ of agonist-induced changes in $[\text{Ca}^{2+}]_i$ was tested by challenging the cells with exogenous ATP which, acting presumably on a phospholipase C-coupled P2y receptor¹⁶, is known to induce large $[\text{Ca}^{2+}]_i$ increases in endothelial cells¹⁷. Addition of 100 μM ATP to a suspension of M4 cells (Fig. 3A, trace a) caused a rapid rise in $[\text{Ca}^{2+}]_i$. When analysed by $[\text{Ca}^{2+}]_i$ imaging, all cells responded to this concentration of ATP, more than 70% of them showing a $[\text{Ca}^{2+}]_i$ rise larger than 500 nM. Trace b shows that ATP also induced a rapid and transient increase in aequorin luminescence. The upstroke of the aequorin signal was almost identical in time course to that of $[\text{Ca}^{2+}]_i$, revealed by Fura-2. In contrast, the decline of the aequorin signal was much faster, with return to basal values within 10 s, whereas $[\text{Ca}^{2+}]_i$ remained elevated at a slowly declining plateau for several minutes. Finally, when

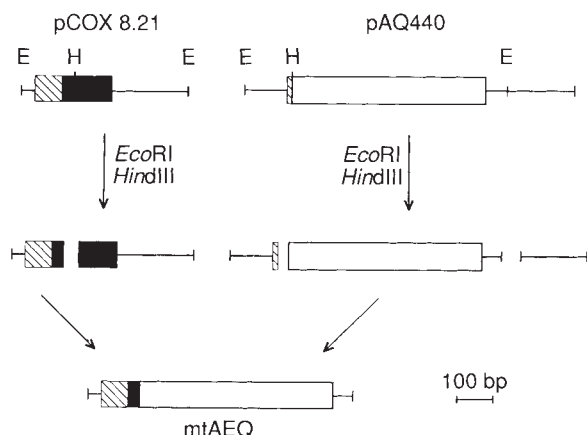


FIG. 1 Construction strategy of the chimaeric mitochondrial aequorin (mtAEQ) cDNA. The cDNA encoding subunit VIII of human cytochrome c oxidase, pCOX8.21 (ref. 10), and the aequorin cDNA¹¹, pAQ440, were digested with the restriction enzymes *EcoRI* and *HindIII*. This allowed the excision of a 150-bp pCOX8.21 fragment (including the 5' non-coding portion, the start codon and the sequence specifying the first 33 amino acids) and a 530-bp AQ440 fragment (including the sequence coding for all the amino acids of the mature polypeptide but the first, the stop codon and part of the 3' non-coding region). The two fragments were ligated together and the resulting cDNA, denominated mtAEQ, encoded a polypeptide composed of the 25 amino acids of COX8 mitochondrial presequence, the first 8 amino acids of COX8 mature protein and virtually the whole photoprotein. The encoded polypeptide includes, in front of aequorin, a complete targeting sequence³² and a portion of the mature mitochondrial protein which should be large enough to include the authentic cleavage site³³ and thus allow the removal of the presequence after the import of the polypeptide. The chimaeric cDNA was subcloned in the expression vector pMT2¹², and the recombinant vector (mtAEQ-pMT2) was used for transfecting the cell lines.

ATP challenged cells were treated with the non-ionic detergent Triton X-100, a large increase in aequorin luminescence was observed, indicating that most of the aequorin was still present within the cells. The results of traces *c* and *d* conclusively demonstrate that the aequorin signal induced by exogenous ATP originates from the mitochondrial matrix and is not due to cytosolic aequorin *en route* to the mitochondria. The addition of the mitochondrial uncoupler carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) almost completely prevented the increase in aequorin luminescence evoked by ATP (trace *d*). At the same time FCCP caused a small increase in $[Ca^{2+}]_i$ and hardly affected that caused by ATP (trace *c*). A drastic reduction of the ATP-induced $[Ca^{2+}]_m$ rise, albeit not as complete as with FCCP, could be observed also when the mitochondrial membrane potential was collapsed by incubating the cells with antimycin A, which blocks the respiratory chain, plus oligomycin, which inhibits the mitochondrial ATPase (not shown).

The conversion of the aequorin signal into absolute $[Ca^{2+}]_m$ values is hampered by several problems; nevertheless, an approximate calculation of the mean $[Ca^{2+}]_m$ can be attempted, as described by Blinks and co-workers^{5,18}, assuming a $[Mg^{2+}]_m$ of 1 mM¹⁹. An upper limit of 200 nM was estimated for $[Ca^{2+}]_m$ under resting conditions. This value agrees closely both with

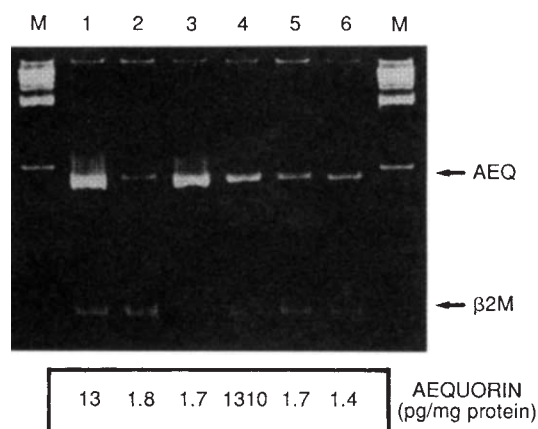


FIG. 2 PCR amplification of mtAEQ sequences from the DNA of the isolated G418-resistant cellular clones and functional evaluation of the aequorin content. The bovine aortic endothelial cell line BAEC was grown in Dulbecco Modified Eagle's Medium, DMEM (supplemented with 10% fetal calf serum, penicillin and streptomycin) in plastic bottles (Falcon). Cells were transfected by the calcium phosphate method with a 9:1 ratio of mtAEQ-pMT2 and pSV2-neo (to allow for selection), according to standard procedures³⁴. After selection, six independent cellular clones were isolated and expanded. Genomic DNA was extracted according to ref. 35. A quantitative PCR, using 1 µg of cellular DNA, was done using two sets of primers: 5'-CCCAAGATGGATTGGACG-3' (sense) and 5'-GCAAGCAGGATCCATGG-3' (antisense), for aequorin (AEQ), and 5'-TTAGCTGTGCTCGCTACTCTCT-3' (sense) and 5'-GTCGGATTGATGAAACCCAGACAC-3' (antisense) for the endogenous gene β_2 -microglobulin (β_2M), used as internal control. M, molecular mass markers (*HindIII*-cut λ DNA); lanes 1–6 identify the different cellular clones. To determine the amount of functional recombinant aequorin produced by each clone, cells (about 1 mg total protein) were trypsinized, washed and resuspended in 200 µl of Ca^{2+} free buffer (150 mM Tris-Cl pH8, 4 mM EGTA and 1 mM PMSF). The cell suspension was freeze-thawed three times. After centrifugation the soluble supernatant was incubated overnight in the presence of 2.5 µM coelenterazine and 150 mM β -mercaptoethanol. Ca^{2+} -dependent aequorin luminescence was measured with a Packard Picolite Model A6100 by adding 10 mM $CaCl_2$. After subtraction of Ca^{2+} -independent background luminescence, the amount of functional aequorin was calculated on the basis of a calibration curve obtained with purified aequorin. The Ca^{2+} affinity of the recombinant aequorin was not appreciably different from that of native aequorin (not shown). The aequorin content of each clone (pg mg⁻¹ of total soluble protein) is the mean of two independent experiments.

TABLE 1 Release of aequorin and marker enzymes by digitonin

	100 µM digitonin (I supernatant)	Freeze-thawing of the digitonin pellet (II supernatant)
Lactate dehydrogenase (% of total activity)	90%	10%
Glutamate dehydrogenase (% of total activity)	<5%	>95%
Aequorin (% of total luminescence)	3%	97%

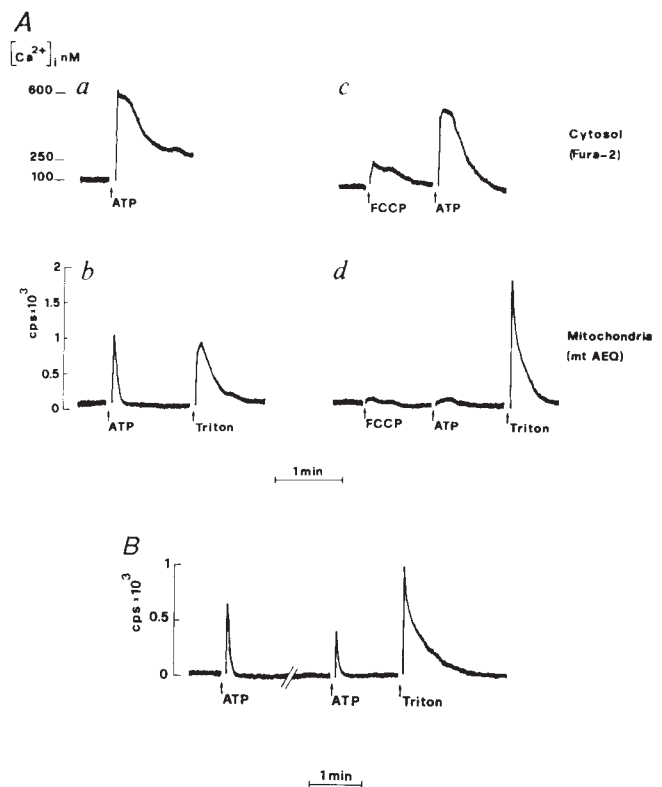
M4 cells were trypsinized, washed and resuspended in Ca^{2+} -free KRB, supplemented with 4 mM EGTA. The cell suspension was incubated at 37 °C and treated with 100 µM digitonin to permeabilize the plasma membrane¹⁵. After centrifugation, the supernatant (I) was collected and the pellet, resuspended in the same buffer, was freeze-thawed three times. After centrifugation, the second supernatant (II) was recovered. Aequorin was reconstituted and measured as described in Fig. 2. LDH and GDH were measured as described previously¹⁵. The values are a mean of two independent experiments.

previous data (ref. 20) and with indirect estimates (ref. 3). On stimulation with ATP, average $[Ca^{2+}]_m$ in the analysed cell suspension increased to above 5 µM in less than 5 s. The sharp drop of the aequorin signal following its rapid rise seems to be due to a real decrease of $[Ca^{2+}]_m$ and not to aequorin consumption. Indeed, cells treated first with ATP, followed by a resting period to allow for consumption of the exogenous ATP by ecto-ATPases²¹, responded with a sharp rise in light output to a second challenge with the same stimulus (Fig. 3B). This experiment indicates that not all aequorin was consumed in the responding cells. Furthermore, if the second addition of ATP was applied shortly after the first, the agonist evoked no aequorin signal. This indicates that the second response is not due to the doubling of the ATP concentration, but to resensitization of the receptor (not shown and see ref. 22).

The data presented provide the first direct measure of changes of $[Ca^{2+}]_m$ induced by physiological stimuli that increase $[Ca^{2+}]_i$. The kinetics and amplitude of the $[Ca^{2+}]_m$ changes are much faster and larger than previously suggested²⁰, possibly due to the minimal Ca^{2+} buffering by aequorin. The experiments (see Fig. 3A) confirm the commonly held view that mitochondria play a minor role in buffering the $[Ca^{2+}]_i$ changes occurring on stimulation of surface receptors. The physiological relevance of these observations seems important; in particular, the results show that during fast changes of $[Ca^{2+}]_i$, possibly including $[Ca^{2+}]_i$ oscillations, parallel rapid changes of $[Ca^{2+}]_m$ occur, thus providing a mechanism for modulating accordingly the activity of mitochondrial enzymes³. The methodology here used is applicable to any cell line and potentially could be used to generate transgenic animals, providing an invaluable tool for investigating even *in vivo* the changes in $[Ca^{2+}]_m$ occurring under physiological and pathological stimuli.

Moreover, the same approach can be extended to the study of Ca^{2+} concentration in the lumen of other cellular compartments. In fact targeting sequences for organelles are known and have been successfully used for directing a number of heterologous proteins^{23,24} to specific subcellular locations. In particular, the nucleus and the endoplasmic reticulum (ER) are our next candidates for being studied by this approach. In the nucleus Ca^{2+} gradients have recently been shown²⁵, and the intranuclear Ca^{2+} concentration has been proposed to be involved in the regulation of different processes such as gene expression²⁶ and apoptosis²⁷. A Ca^{2+} indicator specifically localized in the nucleus could override the technical and theoretical problems so far encountered with the fluorescent dyes. Of utmost interest is also the direct assessment of Ca^{2+} levels in the ER. Not only is this organelle (or some specialized subcompartment) the main source for agonist-mobilizable Ca^{2+} , but the extent of

FIG. 3 A, Effect of extracellular ATP and of the mitochondrial uncoupler FCCP on cytosolic and mitochondrial Ca^{2+} concentrations. B, Effect on $[\text{Ca}^{2+}]_m$ of sequential stimulation with exogenous ATP. METHODS A, Traces a and c, $[\text{Ca}^{2+}]_i$ measured in suspensions of Fura-2 loaded M4 cells. M4 cells were trypsinized, washed and resuspended in complete DMEM culture medium. The cell suspension was incubated overnight in this medium at 4 °C under the same conditions used for reconstituting aequorin (see below). In some experiments coelenterazine was also added, with no appreciable difference in the results. Fura-2 loading and calibration of the signal in terms of $[\text{Ca}^{2+}]_i$ were done as previously described^{22,36}. An aliquot of the cell suspension was centrifuged immediately before use and resuspended in a modified Krebs Ringer buffer, KRB, containing (in mM)⁻¹: 125 NaCl, 5 KCl, 1 MgSO_4 , 1 CaCl_2 , 1 Na_2PO_4 , 5.5 glucose, 20 HEPES (pH 7.4 at 37 °C). Where indicated ATP, 100 μM , and FCCP, 2 μM , were added. FCCP caused a small artefactual quenching of the Fura-2 signal; the trace was corrected graphically. In FCCP-treated cells the plateau of elevated $[\text{Ca}^{2+}]_i$ following ATP addition was almost abolished. This could have a number of explanations, such as depletion of cellular ATP, acidification of the cytosol, depolarization of the plasma membrane potential¹⁵. On the left the mean $[\text{Ca}^{2+}]_i$ values are presented. The experiments were done at 37 °C and are representative of five similar trials. $[\text{Ca}^{2+}]_i$ was measured also by ratio imaging of single, Fura-2 loaded, M4 cells with a system purchased from Analytical Imaging Concepts (Georgia). Traces b and d, $[\text{Ca}^{2+}]_m$ measured by aequorin luminescence. The cells were trypsinized, washed and resuspended as described above. Coelenterazine (2.5 μM) was added and the cells incubated overnight in the dark at 4 °C to allow for reconstitution of aequorin. In each experiment an aliquot of the cells was centrifuged immediately before use, resuspended in KRB and transferred to the luminometer tube. All other conditions are as described above. The experiments presented have been done on at least five different batches of cells, with very similar results. Where indicated, Triton X-100, final concentration 1.5%, was added. ATP, FCCP and Triton X-100, at the concentrations used here, have no quenching effect on the luminescence of purified aequorin; the consumption of aequorin in unstimulated cells is negligible (<1% in 20 min). The calibration of the aequorin signal in terms of $[\text{Ca}^{2+}]_m$ was done essentially as described by Allen and Blinks¹⁸ by integrating the total light emission produced by Triton X-100 addition (corrected for the amount of aequorin consumed by ATP stimulation, when relevant) and calculating the flash that would have been obtained had the aequorin been exposed instantly to saturating Ca^{2+} concentration. The time, after Triton X-100 addition, required to consume all the mitochondrially targeted photoprotein is longer than that necessary to bleach cytosolic aequorin (R.R. *et al.*, unpublished), as expected for a protein sequestered



within the mitochondrial matrix. At these concentrations Triton X-100 produced no spurious signal. Given the amount of aequorin produced by M4 cells, the light emitted by resting cells was very low, thus an upper limit for basal $[\text{Ca}^{2+}]_m$ was estimated by integrating the amounts of light emitted over 15 min, subtracted of the Ca^{2+} -independent luminescence of freeze-thawed cells. B, Conditions as in Fig. 3A, b and d. Where indicated 100 μM ATP and 1.5% Triton X-100 were added. //, Indicates 9 min. interruption of the trace.

its Ca^{2+} filling seems essential for controlling a number of processes, such as $[\text{Ca}^{2+}]_i$ oscillations²⁸, Ca^{2+} influx across the plasma membrane²⁹, as well as protein processing in the lumen³⁰. Although the strategy for targeting aequorin to these compartments appears straightforward^{23,24}, in the ER the measurement could be difficult since, owing to the presumably high Ca^{2+} concentration in its lumen, consumption might override aequorin reconstitution. In this case it might prove useful to change the Ca^{2+} affinity of aequorin by reconstituting the

apoprotein with synthetic analogues of the prosthetic group³¹ or by mutating its Ca^{2+} binding site.

Finally, other potential applications of this methodology, besides the study of Ca^{2+} homeostasis, can be envisaged. The subcellular targeting of a Ca^{2+} sensitive photoprotein should provide a highly sensitive reporter system for the study in intact cells of the regulation of mitochondrial protein import; other complex phenomena such as protein sorting, processing and packaging could be studied *in situ* by this method. □

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Association of tyrosine kinase p56^{lck} with CD4 inhibits the induction of growth through the $\alpha\beta$ T-cell receptor

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THE membrane glycoprotein CD4 enhances antigen-mediated activation of T cells restricted by class II molecules of the major histocompatibility complex (MHC)¹⁻³. This positive function has been attributed to the protein tyrosine kinase p56^{lck} (ref. 4), which is noncovalently associated with the cytoplasmic portion of CD4^{5,6}, and is activated on CD4 aggregation⁷. Antigen presentation by MHC class II molecules coaggregates CD4 and the T-cell antigen receptor (TCR $\alpha\beta$ -CD3)⁸. Thus, the mutual specificity of CD4 and TCR $\alpha\beta$ for the MHC-antigen complex results in the juxtaposition of p56^{lck} and TCR $\alpha\beta$ -CD3⁹⁻¹³. In contrast, anti-CD4 antibodies can abrogate antigen-induced¹⁴⁻¹⁷, as well as anti-TCR-induced¹⁸⁻²⁰ T-cell activation, indicating that CD4 might also transduce negative signals. The molecular basis for this opposing function remains unclear. Here we show that the CD4-p56^{lck} complex prohibits the induction of activation signals through the TCR-CD3 complex when not specifically included in the signalling process. This negative effect does not require anti-CD4 treatment, indicating that the induction of distinct negative signals is probably not involved. Rather, the results demonstrate that the CD4-p56^{lck} complex provides prerequisite signals for antigen-receptor-induced T-cell growth and thus characterize a molecular mechanism for functional constraints imposed on T-cell activation by the MHC.

We have isolated CD4⁺ and CD4⁻ variants of a V β 4-expressing T-cell clone specific for an ovalbumin-derived peptide in association with I-A^b (ref. 21). The CD4⁺ variant clone 2.5 and the CD4⁻ variant clone 2.10 respond comparably to a broad range of antigen concentration (10–100 μ g ml⁻¹). Thus, CD4 neither provides a prerequisite signal, nor needs to function as an adhesion molecule^{15,17} in the antigen-specific response of these cells. Nonetheless, antigen-induced growth of clone 2.5 is inhibited by anti-CD4 antibodies (Table 1a), indicating that antibody-induced aggregation of CD4 may deliver a negative signal¹⁸⁻²⁰. The surprising observation came when assessing the responsiveness of clones 2.5 and 2.10 to anti-TCR $\alpha\beta$ (H57.597, ref. 22), and anti-V β 4 (KT4.10, ref. 23) monoclonal antibodies. Although both clones respond comparably to anti-CD3 ϵ (145.2C11, ref. 24), the responses of the CD4⁺ clone 2.5 to either TCR $\alpha\beta$ - or V β 4-specific antibodies were more than 10-fold lower than those observed with the CD4⁻ clone 2.10 (Table 1a). Because both clones express functional TCR-CD3 complexes (Table 1a), these results suggest that expression of CD4 by clone 2.5 may be responsible for its inability to respond to the TCR-specific antibodies.

To investigate this, we infected clone 2.10 with retroviruses containing the neomycin resistance gene alone, or in combination with mouse complementary DNAs encoding either wild-type CD4 or a mutated CD4 molecule in which the cysteine residues required for association with the cellular tyrosine kinase

p56^{lck} (refs 25, 26) were substituted with alanine residues. Figure 1a illustrates that infection with retrovirus encoding either the double cysteine CD4 mutant (NDC) or wild-type CD4 (NC) resulted in stable levels of CD4 expression ranging from 65% to 100% of that observed on clone 2.5. Lck immunoblots reveal that these clones contain comparable levels of p56^{lck} (Fig. 1b). Further, anti-CD4 coprecipitated comparable levels of p56^{lck} from clone 2.5 and wild-type CD4 infectants of clone 2.10 (Fig. 1b). As previously reported^{25,26}, p56^{lck} did not coprecipitate with CD4 containing the alanine substitutions.

Expression of the neomycin resistance gene in clone 2.10

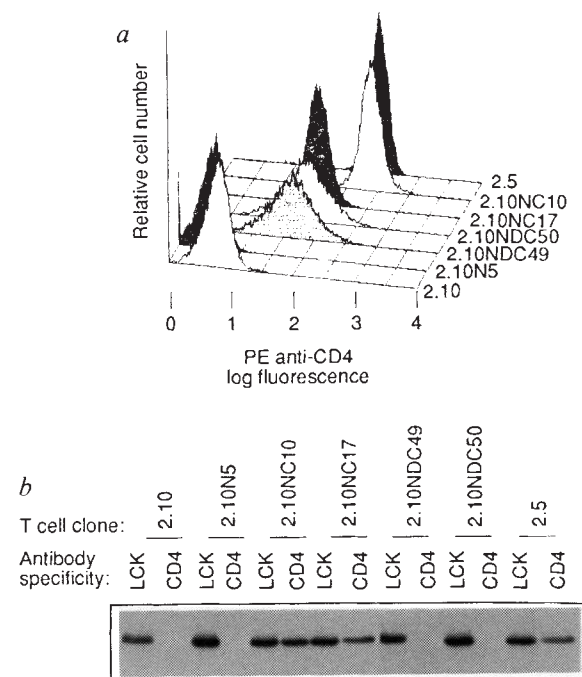


FIG. 1 a, Expression of exogenous CD4 in clone 2.10. b, Differential coprecipitation of p56^{lck} with CD4 variants. a, Wild-type mouse CD4 cDNA (provided by J. Parnes, Stanford University, Palo Alto, California) was subcloned into the retrovirus expression vector, pLXSN (ref. 41). A mutated mouse CD4 cDNA, containing alanine substitutions at residues 418 and 420 was generated using the polymerase chain reaction (PCR) overlap-extension technique as previously described⁴² and subcloned into the retroviral expression vector, MNC Stuffer⁴³. Both pLXSN and MNC Stuffer contain the neomycin resistance gene (neo). DAMP cells⁴³ were transfected with MNC Stuffer containing the mutated CD4 cDNA, and Ψ -2 cells⁴⁴ were transfected with either pLXSN containing no insert or pLXSN containing wild-type CD4 cDNA. Subsequently, where applicable, drug-resistant packaging cells were sorted for high surface expression of CD4. Clone 2.10 was infected with virus containing neo (N), neo-wild-type CD4 (NC), or neo-mutated CD4 (NDC), using either supernatants of sorted, transfected packaging cells⁴¹, or by coculture with mitomycin C-treated, sorted, transfected packaging cells. CD4⁺ infectants were enriched for high-level CD4 expression by cell sorting, and then cloned by micromanipulation or FACSTAR autoclone. Infectants were maintained in medium supplemented with 8 U ml⁻¹ rIL-2 and 500 μ g ml⁻¹ G418. CD4 expression by clone 2.10, neo-infectants (N), neo-wild-type CD4 infectants (NC), and neo-mutated CD4 infectants (NDC) of clone 2.10, as well as clone 2.5, was determined by immunofluorescent staining and FACS analysis using phycoerythrin (PE) conjugated anti-CD4 (GK1.5 Becton Dickinson). b, Lck immunoblots were done as previously described⁵. Briefly, T cells were lysed at 5×10^7 cells ml⁻¹ in lysis buffer containing 1% Nonidet P-40, and postnuclear fractions were prepared by spinning lysates at 13,000g for 10 min. CD4 was precipitated from 5×10^6 cell equivalents using 1 μ g anti-CD4 (H129, ref. 37). Immune complexes were subsequently collected by the addition of *Staphylococcus aureus* (Staph A, Pansorbin, Calbiochem), which had been pre-coated with polyclonal rabbit anti-rat antiserum (RAR, Jackson). Lck was precipitated directly by adding 1 μ l rabbit anti-p56^{lck} (ref. 5) to lysates containing 5×10^6 cell equivalents, and immune complexes collected with Staph A. CD4 and p56^{lck} precipitates were fractionated by SDS-PAGE (8% acrylamide), and transferred to nitrocellulose, blocked, incubated with rabbit anti-p56^{lck}, and developed with ¹²⁵I-labelled protein A.

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infectants did not alter responsiveness to antigen, anti-TCR $\alpha\beta$, or anti-CD3 ϵ (Table 1, Fig. 2a-c). In contrast, the responses to TCR $\alpha\beta$ antibody by all wild-type CD4 infectants of clone 2.10 were virtually abrogated (Fig. 2e), a phenotype identical to that observed in the CD4⁺ clone 2.5 (Table 1). Moreover, as observed with clone 2.5, these wild-type CD4 infectants responded well to both antigen (Fig. 2d) and anti-CD3 ϵ (Fig. 2f), indicating that the TCR-CD3 complex was indeed functional.

To determine directly whether CD4 expression *per se* is sufficient to prohibit the induction of growth by TCR $\alpha\beta$ antibody, we analysed the response of mutated CD4 infectants of clone 2.10 (NDC) to TCR $\alpha\beta$ antibody. As illustrated in Fig. 2h, despite expression levels of CD4 comparable to wild-type CD4 infectants of clone 2.10 (Fig. 2j), mutated CD4 infectants responded as well to anti-TCR $\alpha\beta$ as they did to antigen (Fig. 2g). To emphasize the point, proliferation results obtained from each category of infectant were normalized by creating a ratio of the responses of each clone to TCR $\alpha\beta$ antibody and to antigen. As illustrated in Fig. 2j, plotting this ratio relative to the level of CD4 expression for each clone reveals that both

control (N) and mutated CD4 (NDC) infectants of clone 2.10 respond as well to TCR $\alpha\beta$ antibody as they do to antigen. In contrast, responses of wild-type CD4 (NC) infectants to anti-TCR $\alpha\beta$ range from 1 to 15% of those obtained with antigen. Thus, the capacity of CD4 to prohibit the induction of T-cell growth by TCR $\alpha\beta$ antibody correlates with its association with p56^{lck}.

As suggested by many studies, antigen presented in association with MHC class II molecules would serve to coaggregate CD4 and TCR $\alpha\beta$ -CD3, juxtaposing CD4-associated p56^{lck} and its putatively relevant substrates^{1-3,10-13}. The prediction follows that deliberate antibody-mediated coaggregation of CD4 and TCR-CD3 in clone 2.5 and in wild-type CD4 infectants of clone 2.10 should result in the induction of T-cell growth, as is the case (Table 1b). Rat monoclonal antibody specific for either V β 4, CD4, or a combination of the two were added to culture wells coated with polyclonal mouse anti-rat IgG. Clone 2.10

TABLE 1 Effects of CD4 on T-cell activation

(a) Expression of CD4 correlates with the failure of TCR $\alpha\beta$ antibodies to induce growth

Thymidine incorporation (c.p.m. $\times 10^{-3}$) in response to

Clone	OVA/I-A ^b	OVA/I-A ^b +anti-CD4	Anti-CD3 ϵ	Anti-TCR $\alpha\beta$	Anti-V β 4
2.5 ^{CD4+}	61.3 $\pm$ 2.3	4.6 $\pm$ 0.2	72.0 $\pm$ 7.0	4.7 $\pm$ 0.2	3.1 $\pm$ 0.2
2.10 ^{CD4+}	45.5 $\pm$ 2.6	41.4 $\pm$ 0.5	88.4 $\pm$ 7.6	59.7 $\pm$ 0.9	53.8 $\pm$ 2.0

(b) Induction of growth through TCR $\alpha\beta$ requires its coaggregation with CD4-p56^{lck}

Thymidine incorporation (c.p.m. $\times 10^{-3}$)
in response to

Clone	Anti-CD4	Anti-V β 4	Anti-CD4 +anti-V β 4	Synergy
2.5	0.04 $\pm$ 0.005	0.29 $\pm$ 0.05	88.1 $\pm$ 2.9	266
2.10	0.03 $\pm$ 0.002	16.4 $\pm$ 1.1	19.4 $\pm$ 1.1	1.2
2.10NC10	0.03 $\pm$ 0.003	0.35 $\pm$ 0.05	96.1 $\pm$ 1.1	253
2.10NDC50	0.04 $\pm$ 0.006	127.7 $\pm$ 4.3	118.4 $\pm$ 4.7	0.93

a. Clones were maintained in serum-free Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 8 U ml⁻¹ recombinant IL-2³⁶. Before assay, cells were collected and washed twice in PBS containing 5% heat-inactivated fetal calf serum. T cells (5×10^4) were cultured, in the absence of IL-2, in 0.2 ml IMDM containing 5×10^5 irradiated (2,000 rads) syngeneic (C57BL/6) splenocytes. Cultures were stimulated with 100 μ g ml⁻¹ ovalbumin, in the presence or absence of 2 μ g ml⁻¹ anti-CD4 (H129, ref. 37). Anti-TCR $\alpha\beta$ (H57.597, ref. 22) or anti-CD3 ϵ (145.2C11, ref. 24) were used at 1 μ g ml⁻¹, and anti-V β 4 (KT410.1, ref. 23) at 3 μ g ml⁻¹. At 40 h, cultures received 1 μ Ci ³H-TdR; 6 h later, they were collected onto filter mats and thymidine uptake assessed by liquid scintillation spectroscopy. Values represent the mean of five experiments, with one standard error indicated. Clones 2.5 and 2.10 responded to 100 μ g ml⁻¹ Keyhole Limpet Haemocyanin with 150 $\pm$ 15 c.p.m. and 120 $\pm$ 14 c.p.m., respectively. The addition of 2 μ g ml⁻¹ anti-MHC class II (M5114, ref. 38) inhibited the antigen response of both 2.5 and 2.10, resulting in 120 $\pm$ 10 c.p.m. and 74 $\pm$ 5 c.p.m., respectively. Neither anti-MHC Class I (M1/42, ref. 39) nor anti-CD8 α (53.6.72, ref. 40) inhibited antigen responses. b. Culture plates were precoated for 1 h at 37 °C with 100 μ l 50 μ g ml⁻¹ polyclonal mouse anti-rat antiserum (Jackson). Wells were washed 3-4 times with PBS and blocked for 1 h at 37 °C with a 5% solution of BSA (Fraction V Albumin, Boehringer Mannheim). Wells were then washed and incubated at 37 °C with IMDM containing 0.4 μ g ml⁻¹ anti-CD4 or 0.15 μ g ml⁻¹ anti-V β 4, or a combination of the two. T cells were added (3.5×10^4 cells per well) and cultures incubated for 25-30 h at 37 °C before addition of 1 μ Ci ³H-TdR per well. Six hours later, cultures were collected onto filter mats and thymidine uptake assessed by liquid scintillation spectroscopy. Values represent the mean of three experiments with one standard error indicated.

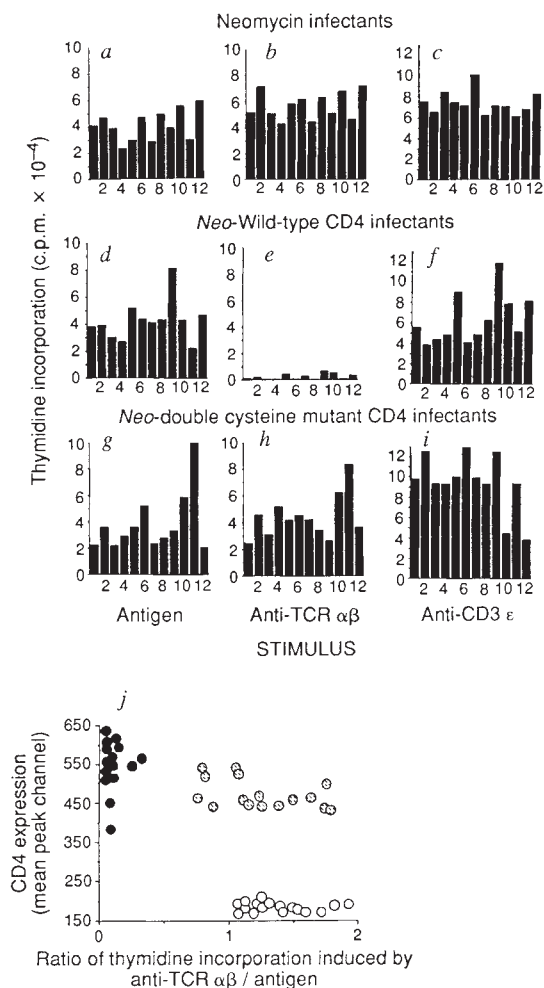


FIG. 2 Association of CD4 with p56^{lck} correlates with lack of response to TCR monoclonal antibody.

METHODS. The *neo* (N), *neo*-wild-type CD4 (NC), and *neo*-mutated CD4 (NDC) infectants of 2.10 were assayed for responsiveness to 100 μ g ml⁻¹ ovalbumin, 1 μ g ml⁻¹ anti-TCR $\alpha\beta$ and 1 μ g ml⁻¹ anti-CD3 ϵ , as described in the legend to Table 1. Responses of 12 representative clones from each category of infection are shown: N (a-c), NC (d-f), NDC (g-i). j, 48 stable, G418-resistant clones have been analysed in this study: 15 2.10 NC clones (filled circles), 16 2.10 NDC clones (flecked circles) and 17 2.10 N clones (circles), were assayed for responsiveness to anti-TCR $\alpha\beta$ and to antigen. Levels of CD4 expression were assessed by immunofluorescence and FACS analysis (see legend to Fig. 1a). The mean peak channel of fluorescence is plotted on the ordinate and the ratio of thymidine incorporation induced by 1 μ g ml⁻¹ anti-TCR $\alpha\beta$ divided by that induced by 100 μ g ml⁻¹ ovalbumin is plotted on the abscissa.

and mutated CD4 infectants of clone 2.10 responded robustly to anti-V β 4 alone. Importantly, the responses of mutated CD4 infectants were not noticeably enhanced in the presence of both anti-V β 4 and anti-CD4. Thus, coaggregation of CD4 *per se* with TCR-CD3 does not provide supplementary activation signals. In contrast, in clone 2.5 and wild-type CD4 infectants of clone 2.10, where p56^{lck} is associated with CD4, the induction of DNA synthesis required coaggregation of CD4 and TCR $\alpha\beta$ (Table 1b).

Calcium ion responses induced through TCR $\alpha\beta$ in clones 2.5, 2.10 and CD4 infectants of clone 2.10, parallel results obtained in proliferation assays (Fig. 3A). Specifically, aggregation of TCR $\alpha\beta$ in the CD4⁻ clone 2.10, and in mutated CD4 infectants of clone 2.10, results in robust mobilization of intracellular Ca²⁺ involving the majority of cells (Fig. 3A, b, g). Responses of mutated CD4 infectants were not enhanced on coaggregation of CD4 and TCR $\alpha\beta$ (compare Fig. 3A, g with h). In contrast, anti-V β 4 stimulation of clone 2.5 and wild-type

CD4 infectants of clone 2.10, results in minimal Ca²⁺ mobilization (Fig. 3A, d, j), which is rescued on coaggregation of CD4 and TCR $\alpha\beta$ (Fig. 3A, e, k).

The induction of tyrosine phosphorylation is the first prerequisite signal induced through the T-cell antigen receptor, without which the induction of subsequent activation signals, including the mobilization of intracellular calcium, are blocked^{27,28}. To assess the role of the CD4-p56^{lck} complex in both prohibiting and enhancing¹² this process, we analysed the accumulation of tyrosine phosphorylated proteins after TCR $\alpha\beta$ aggregation in clones 2.5, 2.10, and CD4 infectants of clone 2.10. Aggregation of TCR $\alpha\beta$ on clone 2.5 (Fig. 3B, a) and on wild-type CD4 infectants of clone 2.10 (Fig. 3B, b) resulted in reduced levels of tyrosine phosphorylation relative to those observed in clone 2.10 (Fig. 3B, a) and mutated CD4 infectants of clone 2.10 (Fig. 3B, b). Quantitative analysis revealed that the levels of phosphorylation of at least four substrates in wild-type CD4⁺ clones were 3–15-fold lower after antigen recep-

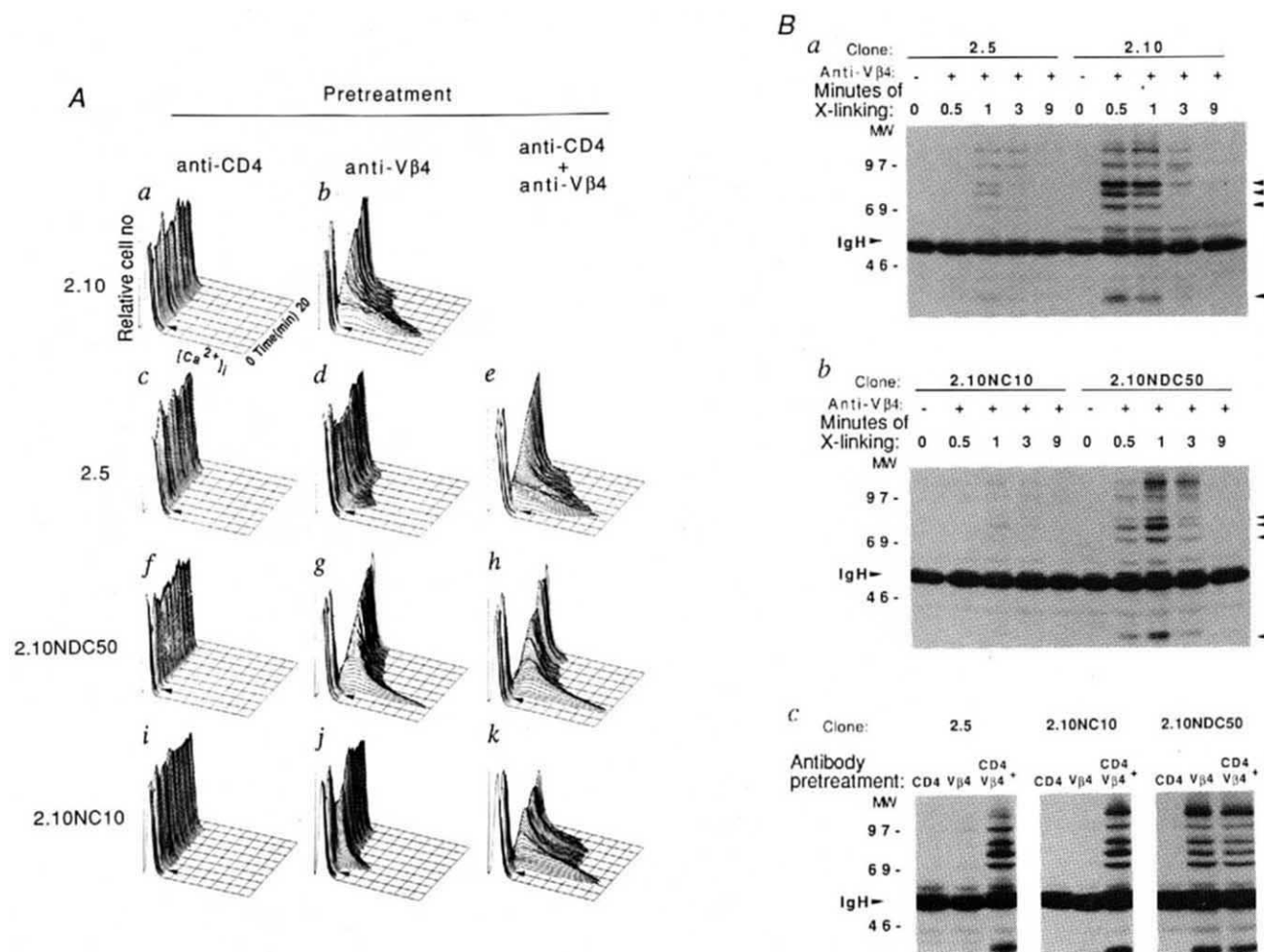


FIG. 3 A, Expression of wild-type CD4 inhibits anti-TCR $\alpha\beta$ induced calcium mobilization. B, Expression of wild-type CD4 inhibits anti-TCR $\alpha\beta$ -induced protein tyrosine phosphorylation.

METHODS. A, Intracellular calcium concentration [Ca²⁺]_i was measured using Indo-1 (Molecular Probes) and a BD FACSTAR plus. Briefly, after loading with Indo-1, cells (at 5×10^6 ml⁻¹) were pretreated with the indicated antibodies specific for CD4 (ref. 37), V β 4 (ref. 23) or a combination of the two, each at $10 \mu\text{g ml}^{-1}$. After washing, cells were passed through the cytometer at a rate of 500 per second to establish a baseline, after which, and indicated by the arrow on each series of histograms, $80 \mu\text{g ml}^{-1}$ polyclonal mouse anti-rat (Jackson) was added. Histograms were generated using BD Lysis II software, plotting [Ca²⁺]_i on the x axis, relative cell number on the y axis, and time (0–20 min) on the z axis. B, Anti-phosphotyrosine immunoblotting has been described⁴. Briefly, in panels (a) and (b), cells at 2.5×10^7 ml⁻¹

were pretreated with antibodies specific for V β 4 (ref. 23), washed, adjusted to 4×10^7 ml⁻¹ and brought to 37 °C. Polyclonal mouse anti-rat antiserum was then added to a final concentration of $40 \mu\text{g ml}^{-1}$. Crosslinking was done for 0.5, 1, 3 and 9 min after which cells were lysed in boiling sample buffer and fractionated on 10% SDS-PAGE. After transfer to nitrocellulose and blocking, phosphotyrosine-containing proteins were revealed by immunoblotting with anti-P-Y antibody (4G10, ref. 45), followed by ¹²⁵I-goat anti-mouse IgG (ICN). The zero time point in a and b represents cells not pretreated with anti-V β 4, but crosslinked with anti-rat IgG for 1 min. c, Cells were pretreated with antibodies specific for CD4, or V β 4, or a combination of the two before crosslinking for 0.5 min. Fractionation and immunoblotting were done as described above. The relative densities of various molecular species shown on immunoblots in a and b, indicated by arrows to the right, were quantitated using a phosphorimager (Molecular Dynamics).

tor aggregation for 30 s (Fig. 3*B*, *a* and *b*, indicated by arrows). As shown in Fig. 3*B*, *c*, coaggregation of TCR $\alpha\beta$ and CD4, which is complexed with p56^{lck}, rescues levels of tyrosine phosphorylation comparable to those observed in mutated CD4 infectants of clone 2.10 stimulated with anti-V β 4 alone.

Thus, the analysis of both early and late antigen receptor-induced signals provides concordant results, which indicate that prerequisite signal(s) are provided by CD4-associated p56^{lck}, and that this complex needs to be close to TCR-CD3. These results can be explained if one considers that in clone 2.5, and in wild-type CD4 infectants of clone 2.10, the majority of cellular p56^{lck} is physically and/or functionally sequestered by CD4 (Fig. 1*b*). This interpretation is consistent with the recent observation that overexpression of wild-type CD4 in mice transgenic for a class I-restricted TCR $\alpha\beta$ inhibited both CD8-dependent proliferative responses, as well as intrathymic positive selection of these class I-restricted T cells^{29,30}.

In contrast to results presented here, we previously reported that anti-TCR $\alpha\beta$ induces robust DNA synthesis in primary CD4⁺ T cells²⁰. An obvious difference is that although the majority of primary CD4⁺ T cells are quiescent, the clones used in this study are propagated and maintained in interleukin-2 (IL-2) before assay. Recent reports indicate not only that IL-2 activates cellular p56^{lck} (ref. 31), but also that p56^{lck} may associate with the β -chain of the IL-2 receptor (IL-2r)³². Not all cellular p56^{lck} is associated with CD4, ranging from 65 to 95% in primary T cells and clones. It is not implausible that non-CD4-associated p56^{lck} in resting primary T cells is available to interact with antibody-induced aggregates of TCR $\alpha\beta$ -CD3, whereas the non-CD4-associated p56^{lck} in clone 2.5 and wild-type CD4 infectants of clone 2.10 may be involved in IL-2r signalling.

Note that previous demonstrations of the functional uncoupling of TCR $\alpha\beta$ and CD3 ϵ in primary mature T cells²⁰ and thymocytes³³ can now be extended to long-term *in vitro* propagated clones (Table 1*a*; Fig. 2). These results are consistent with the notion that p56^{lck} is required to functionally couple TCR $\alpha\beta$ to the CD3 complex. Further, they imply that p56^{lck} may not be involved in regulating signals generated directly through CD3 ϵ , or alternatively, that its involvement is under different functional and/or physical constraints.

In contrast to previous reports analysing the involvement of CD4 in antigen stimulation^{34,35}, we have shown that the loss of CD4 by clone 2.10 does not abrogate its response to antigen. Expression of exogenous wild-type CD4 in clone 2.10 reveals a new functional property of this molecule. CD4 prohibits the induction of growth signals through TCR $\alpha\beta$ in the absence of antigen probably by sequestration of p56^{lck}. This in turn provides a molecular mechanism for the functional constraints imposed on T-cell activation by the MHC complex. □

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Retinoblastoma gene product activates expression of the human *TGF- β 2* gene through transcription factor ATF-2

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THE retinoblastoma susceptibility gene product (pRb) plays an important role in constraining cellular proliferation and in regulating the cell cycle^{1,2}. The pRb inhibits transcription of genes involved in growth control (reviewed in ref. 3) and can regulate transforming growth factor β 1 (*TGF- β 1*) gene expression^{4,5}. *TGF- β* isoforms also down-regulate cellular proliferation^{6,7}. To determine whether pRb also regulates expression of other *TGF- β* isoforms, we examined the effect of pRb on the expression of the human *TGF- β 2* gene. The human *TGF- β 2* promoter contains multiple elements including an ATF site, which is essential for basal promoter activity⁸. Here we report that pRb activates transcription of the human *TGF- β 2* gene. The promoter element responsible for pRb-mediated transcriptional regulation is a binding site for ATF proteins, an extensive transcription factor family⁹. We provide evidence that implicates ATF-2 in pRb-responsiveness. First, the ATF promoter element in the *TGF- β 2* gene is a high-affinity ATF-2-binding site. Second, a GAL4-ATF2 fusion protein can support pRb-mediated transcriptional activation of a promoter containing GAL4-binding sites. Third, ATF-2 in nuclear extracts can interact with pRb. Our results reveal a new mechanism by which pRb constrains cellular proliferation: by activating expression of the inhibitory growth factor, *TGF- β 2*.

A chloramphenicol acetyltransferase (CAT) reporter plasmid containing the human *TGF- β 2* promoter was cotransfected into CCL-64 mink lung epithelial cells with an expression plasmid

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alone (pJ3 Ω) or with this plasmid carrying the human Rb complementary DNA (phRB). Cotransfection of cells with the TGF- β 2-CAT reporter construct (pB2-77) and phRB resulted in induction of CAT activity 12-fold higher than the vector control (Fig. 1, compare lanes 1 and 2).

We next sought to identify the promoter element that mediates responsiveness to pRb. The TGF- β 2 promoter contains an ATF-binding site (5'-GCACGTCA-3') at positions -74 to -67 (Fig. 2). To determine if this ATF site is involved in pRb-mediated transcriptional regulation, we altered it by a two base substitution (5'-GCATGGCA-3'). This mutant promoter (pB2-77mt) was not activated by pRb (Fig. 1, lanes 3 and 4). Likewise, a promoter derivative in which the ATF site was deleted (pB2-40) did not respond to pRb (lanes 5 and 6). This indicates that pRb stimulates transcription of the TGF- β 2 promoter through an ATF-binding site.

To investigate which ATF transcription factor⁹ might be involved in human TGF- β 2 expression, we carried out DNA binding experiments. In a DNA mobility-shift assay a GST-ATF1 fusion protein bound with similar affinities to the ATF sites of the adenovirus (Ad) E2 and the TGF- β 2 promoters (Fig. 2, compare lanes 1 and 3). In contrast, a GST-ATF2 fusion protein bound to the ATF site of the human TGF- β 2 promoter (lane 4) with

higher affinity than to the ATF site of the Ad E2 promoter (lane 2). The same 2 nucleotide (nt) substitution mutant (pB2-77mt), which abolished pRb-responsiveness (Fig. 1), failed to bind GST-ATF2 (Fig. 2b, lane 4). These data indicate that the ATF site in the TGF- β 2 promoter is a relatively high-affinity binding site for ATF-2. Consistent with this conclusion, cotransfection of an ATF-2 expression vector increased basal expression of TGF- β 2 whereas cotransfection of an ATF-1 expression vector did not (data not shown).

To confirm that ATF-2 was involved in pRb-mediated transcriptional activation of TGF- β 2, we designed a protein fusion experiment. Plasmids expressing various GAL4-ATF derivatives were cotransfected with a CAT reporter construct (G5E1bCAT), which contains five GAL4-binding sites upstream from the Ad E1b TATA box¹⁰. To these cotransfection mixtures was added either the human pRb expression plasmid (phRB) or the control expression vector (pJ3 Ω)¹¹. As expected, pRb did not stimulate transcription in the absence of a GAL4 fusion protein (Fig. 3a, lanes 1 and 2) or on cotransfection of the minimal GAL4 DNA-binding domain (data not shown). On cotransfection of GAL4-ATF2, however, transcription was stimulated by pRb (Fig. 3a, lanes 5 and 6). In contrast, transcriptional stimulation was not observed on cotransfection of GAL4-ATF1 (Fig. 3a,

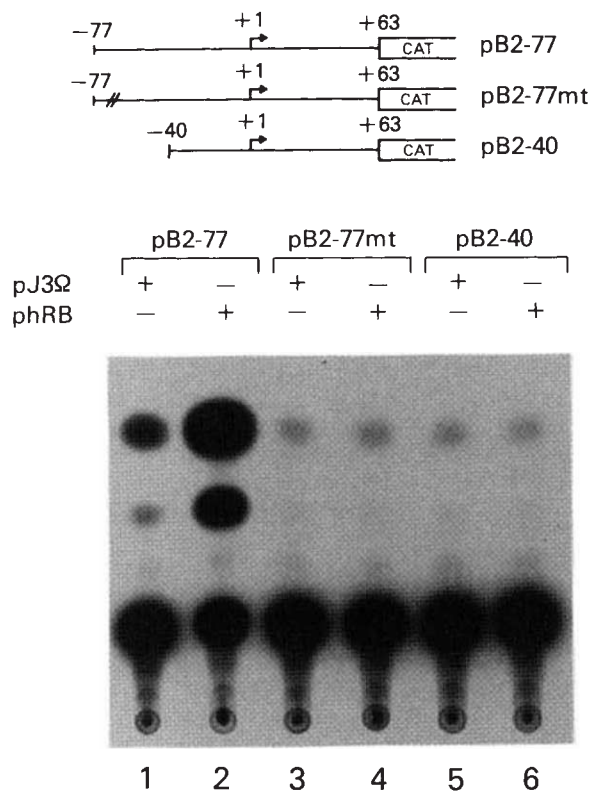


FIG. 1 Effect of pRb on human TGF- β 2 gene expression. An expression plasmid pJ3 Ω (lanes 1, 3 and 5) or the same vector expressing the human Rb complementary DNA (phRB, lanes 2, 4 and 6) was cotransfected with reporter plasmids containing upstream elements of the human TGF- β 2 gene. The constructs pB2-77 and pB2-40 contain promoter sequences -77 to +63 and -40 to +63 linked to the CAT gene, respectively. The mutant construct pB2-77mt has a double point mutation in the ATF site.

METHODS. The construction of reporter plasmids has been described previously⁸. Reporter constructs (pB2-77, pB2-77mt or pB2-40; 10 μ g) were cotransfected with 20 μ g RB plasmid (phRB) or the control plasmid (pJ3 Ω) into CCL-64 cells by the calcium phosphate coprecipitation method. Forty-eight hours after transfection, the cells were collected and CAT activity was determined. Transfection efficiency was normalized by cotransfection of 1.0 μ g of a human growth hormone expression plasmid, pSVGH, and determination of secreted growth hormone in the medium, before assaying for CAT activity (Nichols Institute, San Juan Capistrano, California). Similar results were obtained in three separate experiments.

5'-GGCTGGAGATGACGTAGTT-3' AdE2 ATF
5'-CTAGCACGTCACCTTT-3' -77/-63
5'-CTAGCATGGCCTTT-3' -77/-63 mt

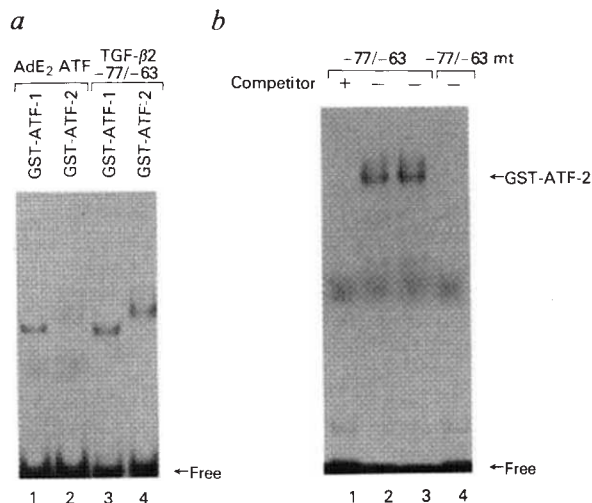


FIG. 2 The ATF site in the human TGF- β 2 promoter is a relatively high-affinity binding site for ATF-2. **a**, ATF-2 preferentially binds to the ATF site in the human TGF- β 2 promoter. Bacterially expressed fusion proteins GST-ATF1 (lanes 1 and 3) and GST-ATF2 (lanes 2 and 4) were used in mobility-shift assays of ATF site-containing oligonucleotides. The adenovirus E2A probe contains sequences from -86 to -68 of the adenovirus E2A promoter. The TGF- β 2 probe is derived from sequences -77 to -63 of the human TGF- β 2 promoter. **b**, Sequence specificity of ATF-2 binding to the TGF- β 2 ATF site. Specificity of GST-ATF2 binding was assayed by competition (50-fold molar excess) with the wild-type TGF- β 2 ATF site -77/-63 (lane 1) or binding to a mutant oligonucleotide -77/-63mt (lane 4). The sequences of the oligonucleotides used are shown.

METHODS. Preparation and purification of the fusion proteins have been described elsewhere¹⁸. Binding reaction mixtures contained 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 5% glycerol, 100 μ M ZnCl₂ and 10,000 c.p.m. ³²P-labelled probe. The oligonucleotide probes used in the gel shift analysis were prepared by annealing the complementary strands and end-labelling using large fragment DNA polymerase I. The analysis of binding complexes was done by electrophoresis on a 5% 0.5X TBE polyacrylamide gel.

lanes 3 and 4) or GAL4-CREB (lanes 7 and 8). GAL4-VP1, an unrelated activator carrying an acidic activating region, activated transcription of G5E1bCAT, but this level of transcription was not stimulated by pRb (lanes 9 and 10). These results indicate that ATF-2 can specifically support pRb-mediated transcriptional activation.

To identify the region(s) of ATF-2 required for pRb-responsiveness, we analysed several GAL4-ATF2 derivatives. Transcriptional activation directed by pRb is progressively diminished on successive deletion of the N-terminal region of ATF-2 (Fig. 3b). Deletion of this same region of ATF-2 also impaired its ability to support transcriptional activation directed by the Ad E1a protein¹².

pRb can negatively regulate transcription of certain cellular and viral genes by forming complexes with the transcription factors required for their expression¹³⁻¹⁷. To investigate whether ATF-2 associates with pRb, we used protein-affinity chromatography. We used a bacterial expression vector in which the glutathione-S-transferase (GST) gene is linked to a DNA fragment encoding a portion of the human retinoblastoma (Rb) gene¹⁸. Mammalian cell nuclear extracts prepared from HeLa and CCL-64 cells were incubated with glutathione-agarose beads containing immobilized GST or GST-Rb¹⁸. Bound proteins were eluted from the column with salt and the presence of ATF proteins in the eluate was assessed by immunoblotting.

Results shown in Fig. 4a validate the specificity of the ATF-2 antiserum used in these experiments. The ATF-2-specific antiserum recognized an *Escherichia coli*-derived GST-ATF2 fusion protein (lane 2) but not other *E. coli*-derived ATF proteins such as GST-ATF1 (lane 3) or GST-ATF3 (lane 4), or the GST moiety itself (lane 1). Conversely, polyclonal antisera raised against GST-ATF1 and GST-ATF3 reacted specifically with ATF-1 and ATF-3, respectively (Fig. 4b, lanes 9 and 12).

Figure 4b shows the results of the protein-affinity chromatography experiments. In nuclear extracts prepared from HeLa (lane 3) or CCL-64 (lane 6) cells, the ATF-2 antiserum reacted with several polypeptides ranging in M_r from ~69 to 90K. As expected, both HeLa and CCL-64 nuclear extracts contain ATF DNA-binding activity (data not shown). GST-Rb retained ATF-2 from both the HeLa (Fig. 4b, lane 2) and CCL-64 (lane 5) extracts as shown by the presence of the ~69K immunoreactive ATF-2 polypeptide in the eluate of the GST-Rb column. In contrast, there was no detectable ATF-2 in the eluate of the GST column (lanes 1 and 4). Figure 4b also shows that binding to the GST-Rb column was relatively specific for ATF-2: neither ATF-1 (lane 8) nor ATF-3 (lane 11), which were both present in nuclear extracts (lanes 9 and 12), were retained on the GST-Rb column. Thus, these results clearly demonstrate that pRb can associate with ATF-2. Whether the interaction between pRb and ATF-2 is direct or involves additional bridging

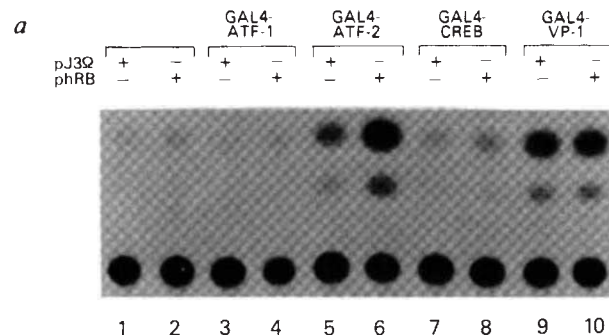
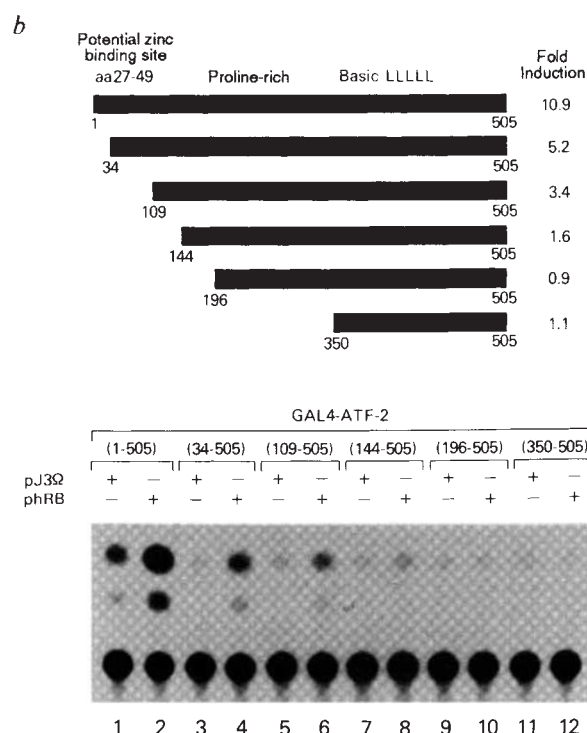


FIG. 3 ATF-2 supports pRb-mediated transcription stimulation. **a**, An expression plasmid for human pRb (pRb, lanes 2, 4, 6, 8 and 10) or the parent vector (pJ3Q, lanes 1, 3, 5, 7 and 9) was cotransfected with a GAL4-reporter plasmid G5E1bCAT and expression vectors for GAL4-ATF1 (lanes 3 and 4), GAL4-ATF2 (lanes 5 and 6), GAL4-CREB (lanes 7 and 8) or the acidic activator GAL4-VP1 (lanes 9 and 10). The reporter construct G5E1bCAT contains 5 GAL4-binding sites upstream of the Ad E1b TATA box and a CAT reporter gene. GAL4-VP1 contains VP16 activator sequences between amino acids 411 and 454 fused to the GAL4 DNA-binding domain, GAL4 (1-147)¹². **b**, pRb-mediated activation is mediated by the N-terminal region of ATF-2. Cotransfections in CCL-64 cells were done with a GAL4 reporter construct, a pRb expression vector and GAL4-ATF2 N-terminal deletion mutants as indicated. A schematic representation of ATF-2 and its derivatives together with the fold-induction by pRb are presented in the upper panel.

METHODS. Construction of GAL4-ATF1, GAL4-ATF2, the GAL4-ATF2 deletions and GAL4-CREB has been described^{12,19}. G5E1bCAT (2 µg), 0.4 µg GAL4-fusion expression vector, and 2 µg of either pJ3Q or pRb were transfected into CCL-64 cells. pSVGH (1 µg) was added as an internal control for transfection efficiency. Each experiment was repeated at least three times.



proteins, and whether the pRb-ATF2 complex binds to the TGF- β 2 promoter, are issues that remain to be determined.

The pRb can repress transcription of specific genes^{11,17}. Here we show that for the TGF- β 2 gene, pRb is a transcriptional activator. It is conceivable that activation of the TGF- β 2 promoter by pRb, like pRb-mediated regulation of the TGF- β 1 promoter⁵, is cell-type specific. Because TGF- β s inhibit proliferation of many cell types and arrest growth in the late G1 phase of the cell cycle^{6,7}, induction of TGF- β 2 by pRb may play an

important role in cell cycle control. Our results also raise the possibility that the cellular target genes of ATF-2 may be involved in growth control. □

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Antisense oligodeoxynucleotides to G_i protein α -subunit sequence accelerate differentiation of fibroblasts to adipocytes

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FULLY-DIFFERENTIATED mouse 3T3-L1 fibroblasts accumulate large amounts of lipid at 7–10 days after induction by insulin or by dexamethasone and a methyl xanthine^{1–3}. G proteins mediate transmembrane signalling from a diverse group of cell-surface receptors to effector units that include phospholipase C, adenylyl cyclase and ion channels^{4–6}. They are also targets of regulation themselves^{7–10}. 3T3-L1 fibroblasts display marked changes in levels of G protein when induced to differentiate to adipocytes^{11–15}. Here we show that cholera toxin, which ADP-ribosylates and activates the G protein subunit G_s, blocks the induction of differentiation, whereas increasing intracellular cyclic AMP directly with the dibutyryl analogue or indirectly with pertussis toxin or forskolin does not affect differentiation. Oligodeoxynucleotides antisense to the sequence encoding G_s accelerate differentiation markedly. The time course of adipogenesis declined from 7–10 days in controls to roughly 3 days in cultures treated with antisense-G_s oligodeoxynucleotides, whereas oligodeoxynucleotides, antisense to G_{i1}, G_{i2}, and sense and missense to G_s, had no such effect. Antisense-G_s alone induced differentiation by day 7, indicating that G_s activity modulates differentiation in 3T3-L1 cells, acting in a new role which is independent of increased intracellular cAMP.

Differentiation of 3T3-L1 fibroblasts to adipocytes that has

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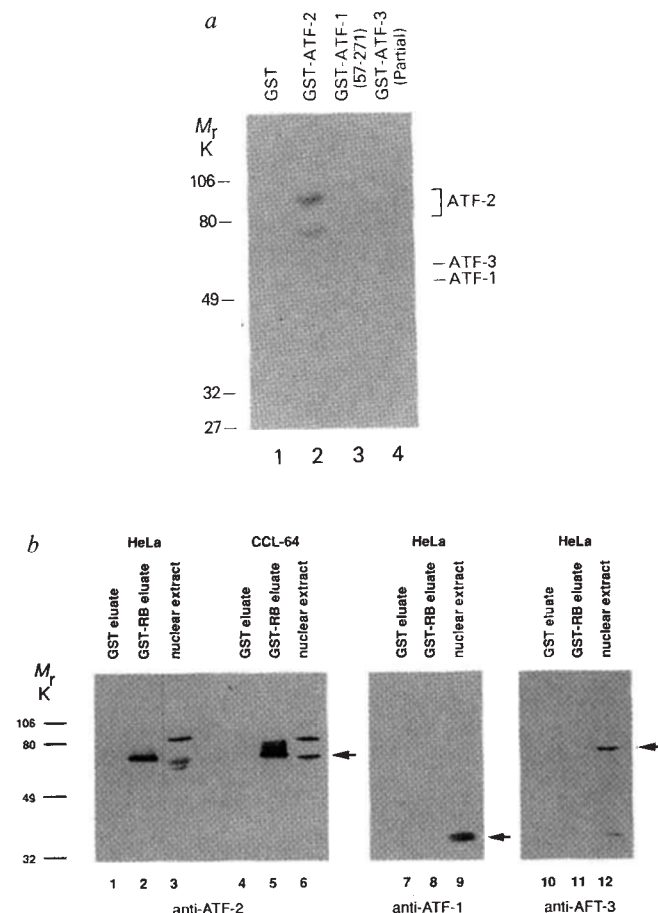


FIG. 4 ATF-2 can associate with pRb. *a*, Specificity of the anti-ATF-2 antibody. Specificity of the ATF-2 antiserum was assessed by immunoblotting against *E. coli*-derived GST (lane 1), GST-ATF2 (lane 2), GST-ATF1 (amino acids 57–271) (lane 3) and GST-ATF3 (partial) (lane 4). The approximate sizes of the GST fusion proteins are indicated at the left. *b*, ATF-2 binds to a GST-Rb column. Nuclear extract from HeLa (lanes 1–3 and 7–12) or CCL-64 cells (lanes 4–6) was incubated with an affinity matrix containing immobilized GST (lanes 1, 4, 7 and 10) or GST-Rb (lanes 2, 5, 8 and 12) and bound proteins detected by immunoblotting with specific antisera to ATF-2 (lanes 1–6), ATF-1 (lanes 7–9) or ATF-3 (lanes 10–12). The position of each ATF protein is indicated by an arrow.

METHODS. Purified proteins (200 ng)²⁰ from different GST-ATF fusions were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with polyclonal antiserum raised against bacterially expressed ATF-2. To generate a pRb-affinity matrix a GST-Rb fusion protein (pGT-RB(379–792)) was expressed in XA90 cells and purified as described¹⁸. The affinity matrix was prewashed in elution buffer, subsequently equilibrated in NETN buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and incubated with nuclear extract²¹ in NETN buffer. Bound proteins were eluted in NETN with 500 mM NaCl. Antiserum specific for ATF-2 (Berkeley Antibody Company) was raised against a partially purified^{22,23} ATF-2 protein expressed from a full-length ATF-2 cDNA in a T7 vector (pET-3C). Rabbit anti-ATF-1 and anti-ATF-3 antisera were produced against GST-fusion proteins containing amino acids 1–30 of ATF-1 and 98 amino acids from a partial ATF-3 cDNA clone, respectively. Antibodies directed against GST were removed by pre-absorption with immobilized GST protein.

been induced by dexamethasone (DEX) and methylisobutylxanthine (MIX) occurs within 7–10 days, when cultures show >95% conversion to cells accumulating lipid droplets (Fig. 1a, b). To investigate the possible role of G proteins in differentiation, we tested the effects of bacterial toxins on the differentiation of fibroblasts induced by DEX/MIX. Bacterial toxins covalently modify α -subunits of G proteins by ADP-ribosylation. Cholera toxin ADP-ribosylates and constitutively activates G_{sa} , the stimulatory G protein coupled to adenylyl cyclase. Cholera toxin was found to block differentiation. Cultures treated with toxin in combination with DEX/MIX did not differentiate (Fig. 1c). Treatment with pertussis toxin, in contrast, did not prevent DEX/MIX from inducing differentiation of 3T3-L1 fibroblasts to adipocytes (Fig. 1d). Although both agents increase intracellular AMP levels, cholera toxin blocks differentiation, whereas pertussis toxin does not. Increasing cAMP concentration through activation of adenylyl cyclase by forskolin (Fig. 1e) or by the direct addition of dibutyryl cAMP to the cultures, in contrast, did not block adipogenesis (Fig. 1f). The ability of cholera toxin rather than elevated cAMP *per se* to block differentiation implies that there is a G_{sa} activity independent of the activation of adenylyl cyclase. Steady-state levels of G_{sa} decline in 3T3-L1 cells during adipogenesis¹⁵. Metabolic labelling of the cultures with [³⁵S]methionine and immune precipitation of G_{sa} show a decline in the synthesis of G_{sa} during differentiation when the first and third days after induction with DEX/MIX are compared (Fig. 2a). Thus, G_{sa} synthesis declines during differentiation and constitutive activation of G_{sa} in cultures by cholera toxin blocks differentiation.

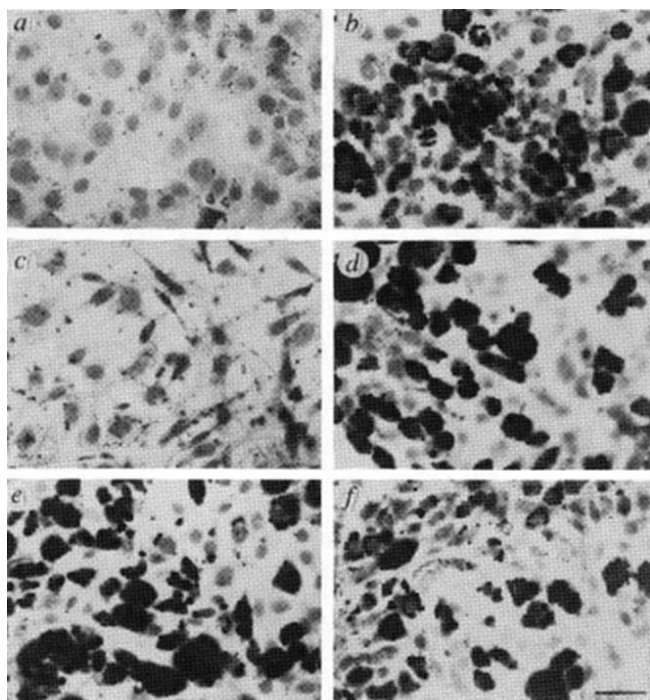


FIG. 1 Cholera toxin blocks differentiation of 3T3-L1 fibroblasts to adipocytes. Mouse embryo fibroblast 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in culture in 100-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Protocols for cell growth and differentiation have been described¹⁵. The day when cells reached confluence is referred to as day 0. Confluent cultures of fibroblasts (day 0) were either untreated (a), treated with dexamethasone (DEX) and methylisobutylxanthine (MIX) for 48 hr (b), or with DEX/MIX in combination with 10 ng ml⁻¹ cholera toxin (c), 10 ng ml⁻¹ pertussis toxin (d), 10 μ M forskolin (e), or 1 mM dibutyryl cAMP (f) for 2 days. Cells were then cultured to day 7 post-confluence with no additions, fixed, stained with oil-red-O and examined by light microscopy. Scale bar, 25 μ m. Results shown are from a single experiment representative of five separate experiments.

We investigated whether suppression of G_{sa} levels would influence differentiation of 3T3-L1 fibroblasts to adipocytes using antisense oligodeoxynucleotides. We synthesized oligodeoxynucleotides complementary to either the sense or the antisense strand of the 20 nucleotides encoding G protein α -subunits immediately 5' to and including the initiator codon ATG (see Fig. 2 legend). Treating 3T3-L1 fibroblasts with oligodeoxynucleotides antisense to G_{sa} resulted in a dramatic reduction (>90%) in the steady-state expression of G_{sa} (Fig. 2b; compare lane 2 for antisense oligomers with lane 1 for control). Oligodeoxynucleotides sense to G_{sa} , in contrast, failed to alter the steady-state expression of this G protein subunit (Fig. 2b; compare lane 3 for sense oligomers with lane 1 for control).

The effects of oligomers antisense to G_{sa} , $G_{i\alpha 1}$, and $G_{i\alpha 3}$ on the differentiation of 3T3-L1 fibroblasts were investigated (Fig. 3). Confluent cultures were treated in chamber slides (0.1 ml) with 30 μ M oligomers in serum-free medium. After 30 min of exposure to oligomers under these conditions, the medium was supplemented with serum (to 10%) and the incubation continued for 24 hours. Treatment with oligomers antisense to G_{sa} accelerated the time course for differentiation induced by DEX/MIX

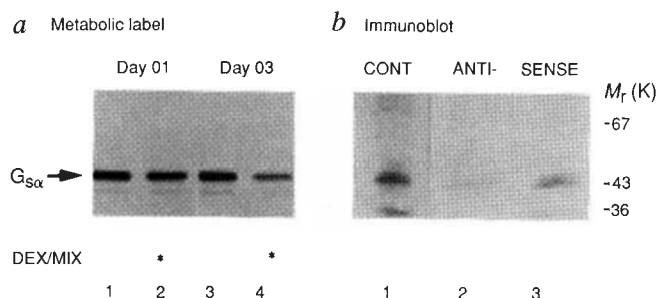


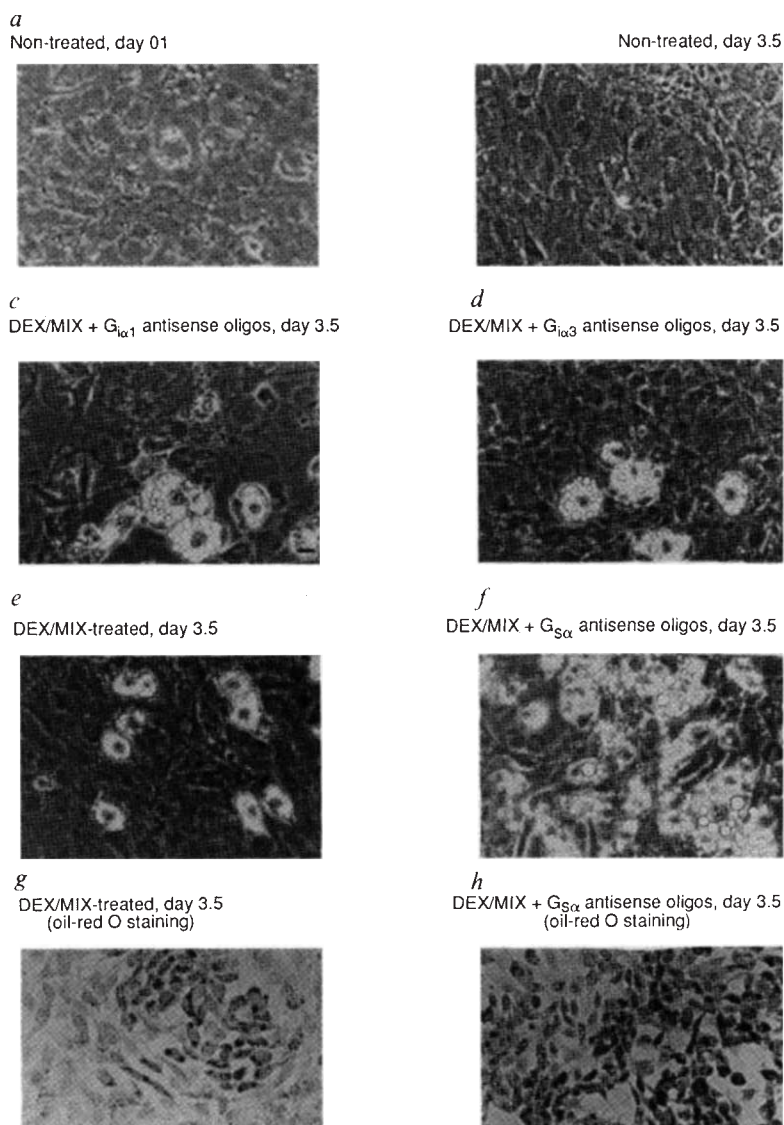
FIG. 2 G_{sa} levels decline during differentiation of 3T3-L1 fibroblasts to adipocytes or by treating cells with oligodeoxynucleotides complementary to G_{sa} sequences. a, For metabolic labelling, cells ($50\text{--}100 \times 10^6$) were washed twice with phosphate-buffered saline and resuspended in DMEM (methionine-free) containing 10% fetal bovine serum supplemented with 200 μ Ci [³⁵S]-methionine (1,000 Ci mmol⁻¹, New England Nuclear) per 10^7 cells. Cells were metabolically labelled with [³⁵S]methionine for 24 h ending with day 1 or day 3 of post-confluent growth of untreated (lanes 1 and 3) and DEX/MIX-treated (lanes 2 and 4, indicated by an asterisk) cells. At the end of the one-day labelling period aliquots of the cells were taken, washed, and membranes prepared from each group of cells. Immunoprecipitations of metabolically labelled G_{sa} were performed from aliquots of detergent-solubilized membranes²¹. Immunoprecipitates were electrophoresed on SDS-polyacrylamide gels and fluorographed. G_{sa} subunits ($M_r \sim 45,000$) are indicated by arrow. Results are from a single experiment duplicated once with identical results. b, The strategy for design and use of the antisense probes was adapted from ref. 22. Synthetic 20-base oligodeoxynucleotides that are 5' to but include the ATG initiator codon of the cDNAs for rat G_{sa} were used in the antisense orientation. These were SGs-20 (G_{sa} sense), 5'-CGCGCCCCGCGCCGCCATG-3'; MSGs-20 (G_{sa} missense), 5'-GGGTCGGCGCGAGCGCGG-3'; ASGs-20 (G_{sa} antisense), 5'-CATGGCGCGCGGGGCGCG-3'; ASGI1-20 ($G_{i\alpha 1}$ antisense), 5'-CATGGTGGCGGAGCGTCGCC-3'; and ASGI3-20 ($G_{i\alpha 3}$ antisense), 5'-CATGACGGCGCGGAGAGG-3. Cells were grown as usual but in Lab-Tek chamber slides (Nunc) to minimize consumption of oligodeoxynucleotides. At one day before confluence, medium was removed and the oligodeoxynucleotides (CONTROL, no oligodeoxynucleotides, lane 1; ANTIsense to G_{sa} , lane 2; SENSE to G_{sa} , lane 3) added in DMEM to a final concentration of 30 μ M. After a 30-min incubation at 37 °C, fetal bovine serum was added to 10% and the incubation continued for 48 h. At the end of the 24-h period confluence was achieved and cultures were then treated with or without dexamethasone and methylisobutylxanthine as described. SDS-polyacrylamide gel electrophoresis of 1 μ g total membrane protein using the PHAST system (Pharmacia) for immunoblotting and staining with antibodies against G_{sa} (ref. 15) showed that synthesis was reduced of G_{sa} targeted with antisense oligomers (lane 2), but not with sense oligomers (lane 3). G_{β} subunit staining of the blots was similar in control, anti-sense and sense G_{sa} -oligomer treated cells (not shown). M_r s of protein markers are shown on the right.

(Fig. 3). Note that few foci of adipogenic conversion are apparent in the DEX/MIX-treated (Fig. 3e, g) as compared to non-treated cells at day 3.5 (Fig. 3b). Cultures treated with DEX/MIX in combination with antisense oligomers to $G_{s\alpha}$, in contrast, were fully differentiated by day 3.5 (Fig. 3f, h). Note the extensive lipid accumulation in cultures treated with antisense- $G_{s\alpha}$ oligomers. Oil-red-O staining of lipid droplets reveals lipid accumulation in 3.5-day cultures of 3T3-L1 cells induced by oligomers antisense to $G_{s\alpha}$ with DEX/MIX (Fig. 3h) as compared to cells induced by DEX/MIX alone (Fig. 3g). Oligomers antisense to $G_{i\alpha1}$ and to $G_{i\alpha3}$, however, did not alter the induction of adipogenic differentiation in response to DEX/MIX (Fig. 3c, d). Lipid accumulation in fixed intact cells was quantified after staining with oil red O (see legends to Figs 3 and 4). These data demonstrate that oligomers antisense to, but not sense to $G_{s\alpha}$ enhance the rate of differentiation induced by DEX/MIX or that observed in the absence of other exogenous inducers. Oligomers antisense, but not sense, to $G_{s\alpha}$ dramatically enhanced the rate and extent of DEX/MIX-induced adipogenic conversion of the cultures (not shown). The ability of cholera toxin to block DEX/MIX-induced differentiation was progressively lost after treatment with oligomers antisense to $G_{s\alpha}$ (30 μ M; data not shown). We investigated next whether oligomers antisense to $G_{s\alpha}$ in the absence of DEX/MIX could by themselves induce differentiation. In cultures that are

7-days post-confluence, oil-red-O staining revealed enhanced lipid accumulation in antisense- $G_{s\alpha}$ oligomer-treated (Fig. 4e, f) as compared with either control, untreated (Fig. 4a, b) or sense- $G_{s\alpha}$ oligomer-treated (Fig. 4c, d) cultures. Enlargement of the fields highlights the differential effects of the oligomers antisense (Fig. 4f), as compared with sense (Fig. 4d), to $G_{s\alpha}$ on the adipogenic conversion of the 3T3-L1 cells. Oligomers missense to $G_{s\alpha}$, like the antisense oligomers, failed to influence differentiation (not shown).

The G proteins represent but one class of a much larger GTPase superfamily whose roles in cellular functions are very diverse⁴⁻⁶. Evidence localizing the G protein G_O to growth cones of neurite outgrowths in nerve growth factor-treated pheochromocytoma (PC12) cells suggests that members of the G protein family may participate in cellular functions other than transmembrane signalling¹⁶. G protein expression changes during differentiation of 3T3-L1 fibroblasts¹¹⁻¹⁵. 3T3-L1 cells differentiate to a phenotype similar to mature adipocytes, a process dramatically accelerated by insulin², dexamethasone¹⁷ and methylxanthine^{17,18}, or growth in high serum concentrations¹⁷. The following observations are indicative of a new and important type of $G_{s\alpha}$ activity, namely the modulation of differentiation: (1) $G_{s\alpha}$ synthesis declines early in differentiation; (2) constitutive activation of $G_{s\alpha}$ by cholera toxin blocks differentiation; (3) oligodeoxynucleotides antisense to $G_{s\alpha}$, but

FIG. 3 Oligodeoxynucleotides antisense to $G_{s\alpha}$, but not $G_{i\alpha2}$ or $G_{i\alpha3}$, accelerate differentiation of 3T3-L1 fibroblasts to adipocytes. Cells were grown in Lab-Tek chamber slides as detailed in Fig. 2 legend. At one day before confluence, medium was removed and oligodeoxynucleotides added in DMEM to a final concentration of 30 μ M. Following a 30-min incubation at 37 °C, fetal bovine serum was replaced to 10% and the incubation continued for 24 h. At the end of this period confluence was achieved and the cultures were then treated with or without dexamethasone and methylisobutylxanthine. Untreated cultures of fibroblasts at day 1 and day 3.5 are shown in a and b, respectively. Cultures induced to differentiate with DEX/MIX alone (e, g) or with DEX/MIX in combination with oligodeoxynucleotides antisense to $G_{s\alpha}$ (f, h), $G_{i\alpha1}$ (c), or $G_{i\alpha3}$ (d) are shown at day 3.5 post-confluence. In a–f, cells were cultured to the day of post-confluence growth indicated, fixed and examined by phase-contrast microscopy. Scale bar, 25 μ m. g and h, Light micrographs of cells fixed and stained with oil-red-O. The results shown are from a single experiment representative of four to six separate experiments. Cells were scored by staining for nuclei with haematoxylin and lipid droplets with oil-red-O. Fields of 50–100 cells were screened at random and the per cent of differentiated cells (positive for lipid droplets) was assessed for each condition. Control, untreated cells at day 3.5, 0% differentiation ($n=4$); cells treated with DEX/MIX at day 3.5, 11.7 $\pm$ 1.7% differentiation ($n=6$); cells treated with DEX/MIX plus oligomers antisense to $G_{s\alpha}$ at day 3.5, 34.7 $\pm$ 2.4% differentiation ($n=6$); and cells treated with DEX/MIX plus oligomers sense to $G_{s\alpha}$ at day 3.5, 13.5 $\pm$ 1.7% differentiation ($n=6$). * $P \leq 0.05$ for difference from DEX/MIX alone.



not those antisense to $G_{i\alpha 1}$, $G_{i\alpha 3}$ or sense to $G_{s\alpha}$, accelerate differentiation, reducing the time to full adipocyte conversion from 7–10 to less than 3 days; and (4) oligodeoxynucleotides antisense, but not sense, to $G_{s\alpha}$ alone are capable of inducing differentiation.

The inability of the dibutyryl analogue of cAMP to block differentiation argues against a role for cAMP in modulating differentiation of 3T3-L1 fibroblasts. Earlier work showing that adipogenesis could be accelerated by treatment with methylxanthines, but not by increasing intracellular cAMP, also argues against adenylyl cyclase functioning as the effector for $G_{s\alpha}$ in this process³. In addition to adenylyl cyclase, Mg^{2+} transport¹⁹ and Ca^{2+} channels²⁰ have been implicated as effector units regulated by $G_{s\alpha}$. Insulin, like the oligomers antisense to $G_{s\alpha}$, accelerates differentiation of 3T3-L1 fibroblasts to adipocytes, perhaps reflecting the ability of insulin to oppose responses, such as lipolysis, mediated by $G_{s\alpha}$. These observations support

a possible new role for G proteins in such complex biological responses as cellular differentiation. □

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Tyrosine-containing motif that transduces cell activation signals also determines internalization and antigen presentation via type III receptors for IgG

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TYPE III receptors for IgG (FcγRII; ref. 1), high-affinity IgE receptors (FcεRI; ref. 2), as well as the T- and B-cell antigen receptors^{3,4}, consist of multiple components with specialized ligand-binding and signal transduction functions^{5–10}. FcγRII α (ligand-binding) and γ (signal-transducing) subunits are expressed in macrophages¹, a cell type involved in the uptake of antigen, its processing and the presentation of the resulting peptides to major histocompatibility complex class II-restricted T lymphocytes^{11,12}. Here we show that murine FcγRIII, transfected into FcγR-negative antigen-presenting B-lymphoma cells, mediate rapid ligand internalization and strongly increase the efficiency of antigen presentation when antigen is complexed to IgG. Efficient internalization and antigen presentation via FcγRIII did not require the cytoplasmic domain of the ligand-binding α-chain, but did require the γ-subunit. Using chimaeric molecules, we show that γ-chain contains a signal for receptor internalization and that the mutation of either of the two tyrosine residues present in its cytoplasmic domain prevents efficient internalization and antigen presentation of immune complexes. Thus, associated chains and their tyrosine-containing motif are not exclusively involved in cell activation, but also determine multimeric receptor internalization.

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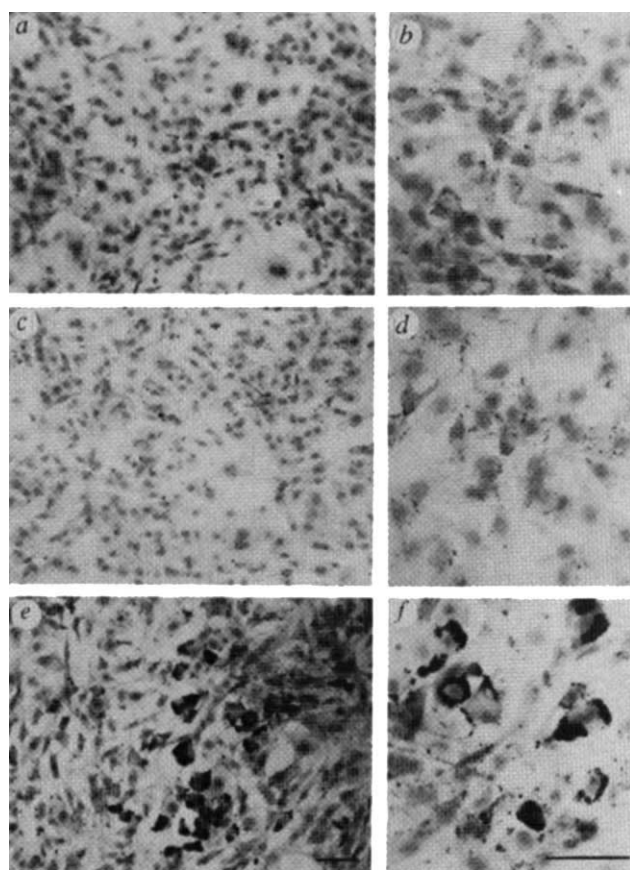


FIG. 4 Oligodeoxynucleotides antisense, but not sense, to $G_{s\alpha}$ alone induce 3T3-L1 fibroblasts to differentiate to adipocytes. Cell growth and treatment with oligomers were as described in Fig. 3 legend, except that cells were cultured to day 7 post-confluence. Differentiation was detected by staining of lipid droplets of fixed cells with oil-red-O. Cultures of 3T3-L1 fibroblasts were untreated (a, b), or treated with oligodeoxynucleotides sense to (c, d), or antisense to (e, f) $G_{s\alpha}$ and examined by light microscopy at 7 days post-confluence. Enlargements of a, c and e displayed as b, d and f, respectively, highlight the lipid accumulation of the cells made visible by oil-red-O staining. Scale bar, 50 μm. Results shown are from a single experiment, representative of 5 separate experiments. Cells were scored by staining for nuclei with haematoxylin and lipid droplets with oil-red-O. Fields of 50–100 cells were screened at random and the per cent cells differentiated (positive for lipid droplets) assessed for each condition. Control, untreated cells at day 7.0, $0.18 \pm 0.7\%$ differentiation ($n=5$); cells treated with DEX/MIX, $90.1 \pm 1.1\%$ differentiation ($n=5$); cells treated with oligomers antisense to $G_{s\alpha}$, $80.0 \pm 2.5\%$ differentiation ($n=5$); and cells treated with oligomers sense to $G_{s\alpha}$, $0.22 \pm 0.9\%$ differentiation ($n=5$). * $P \leq 0.05$ for difference from control, untreated cells.

TABLE 1 cDNA sequence and Fc γ R expression in IIA1.6-transfected cells

cDNA	Sequence	Transfectant cells	Number of receptors per cell ($\times 10^4$)
Fc γ RIIb2	β ct1 ↓ KKKQVPNPDLLEAAKTEAENTITYSLKHPEALDEETEDYQNH	Fc γ RIII (α , γ 2)	2.8
	α ct4 ↓ RRNLQTPREYWRKSLRSIRKHQAPQDK	Fc γ RIII (α ct4, γ 2)	1.4
Fc γ RIII α	α ct4 ↓ RRNLQTPREYWRKSLRSIRKHQAPQDK	Fc γ RIII (α ct4)	1.4
	β ct1 ↓ RRNLQTPREYWRKSLRSIRKHQAPQDK	Fc γ RII (β /ct γ)	10
Fc γ RIII γ	β ct1 ↓ RRNLQTPREYWRKSLRSIRKHQAPQDK	Fc γ RII (β /ct γ Y-L32)	8
	α ct4 ↓ RRNLQTPREYWRKSLRSIRKHQAPQDK	Fc γ RII (ct1)	5
		Fc γ RIIb2	16

Amino-acid sequence (one-letter code) of the cytoplasmic tail of Fc γ RIIb2, Fc γ RII α - and γ -chains and levels of surface expression of transfectant cells. Residues are numbered from left to right and position 1 corresponds to the first presumed cytoplasmic residue. Arrows indicate the positions where stop codons were introduced in β ct1 and α ct4 constructs, the position of the mutated tyrosine residues in the chimaeric constructs (β /ct γ Y-V21 and β /ct γ Y-L32), as well as the junction between Fc γ RII and the cytoplasmic tail of the γ -chain in the chimaeric receptors (β /ct γ). All the mutated and chimaeric receptors were constructed by recombinant polymerase chain reaction as described¹⁰. The cDNAs encoding wild-type, mutated or chimaeric Fc γ RII and Fc γ RIII α -chains were inserted in the simian virus 40-driven vector pKC3, and cDNA encoding the γ -chain of Fc γ RIII was inserted in the cytomegalovirus-driven vector pRc/CMV, which also contained the neomycin-resistance gene. Stable transfectants were obtained by electroporation²¹ and selection into Geneticin-containing medium (G418, 1 mg ml⁻¹; GIBCO). Clones were isolated and checked for Fc γ R expression as described¹⁷. The number of Fc γ R per cell was measured by determining the binding of radiolabelled F(ab')₂ fragments of the monoclonal antibody 2.4G2.

FIG. 2 Antigen presentation by cells expressing Fc γ RIII or β /ct γ chimaeric receptors. Antigen presentation was assayed by culturing non-transfected or transfected IIA1.6 cells together with the λ -repressor-specific T-cell hybridoma 24.4 (ref. 17) for 18–20 h in the presence of various concentrations of the N-terminal 1–102 fragment of λ -repressor complexed (filled circles) or not (circles) with two anti-repressor IgG1 monoclonal antibodies, 22D and 51F (ref. 23), which recognize distinct epitopes (15 μ g ml⁻¹ each IgG). The release of IL-2 by the 24.4 cells into the culture supernatants was determined using a CTL.L2 proliferation assay²⁴. The panels depict λ repressor presentation by receptor-negative untransfected IIA1.6 cells and cells transfected with Fc γ RIII α - and γ -cDNA, Fc γ RIII α ct4 construct, Fc γ RIII α ct4 construct and γ -cDNA, Fc γ RIIb2 cDNA, Fc γ RII chimaera construct (β /ct γ), Fc γ RII tail minus (β ct1) construct, tyrosine-mutated Fc γ RII chimaera construct (β ct γ Y-L32). Each point represents the average of duplicate samples which varied by less than 10%.

METHODS. Culturing of the 24.4 T-cell hybridomas and antigen-presentation assays were done as described¹⁷. Briefly, T-hybridoma cells (24.4) and wild-type or transfected IIA1.6 cells were cultured for 24 h in the presence of various concentrations of soluble or IgG-complexed λ -repressor. Complexes were formed before use by incubating different concentrations of purified fragment 1–102 of λ -repressor, with 15 μ g ml⁻¹ of each of two different purified anti- λ repressor mAb (51F and 22D) at 37 °C for 15 min.

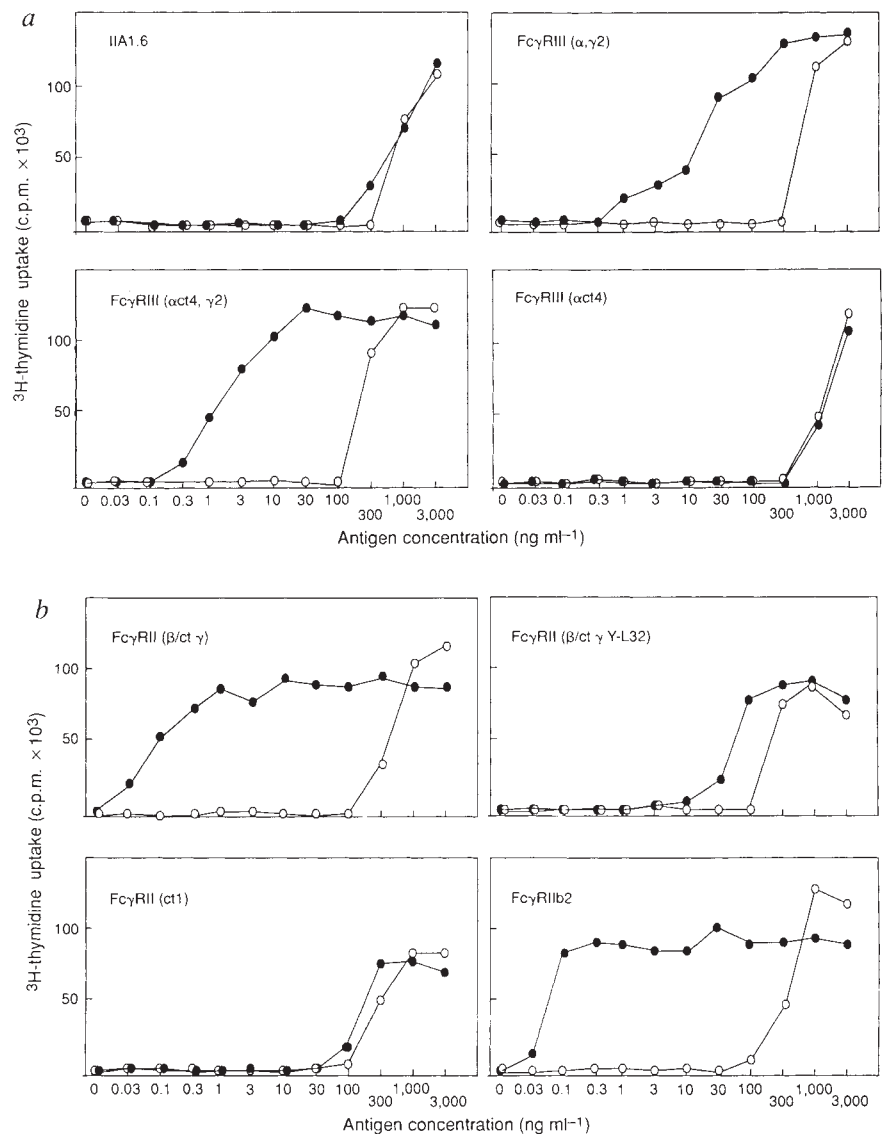
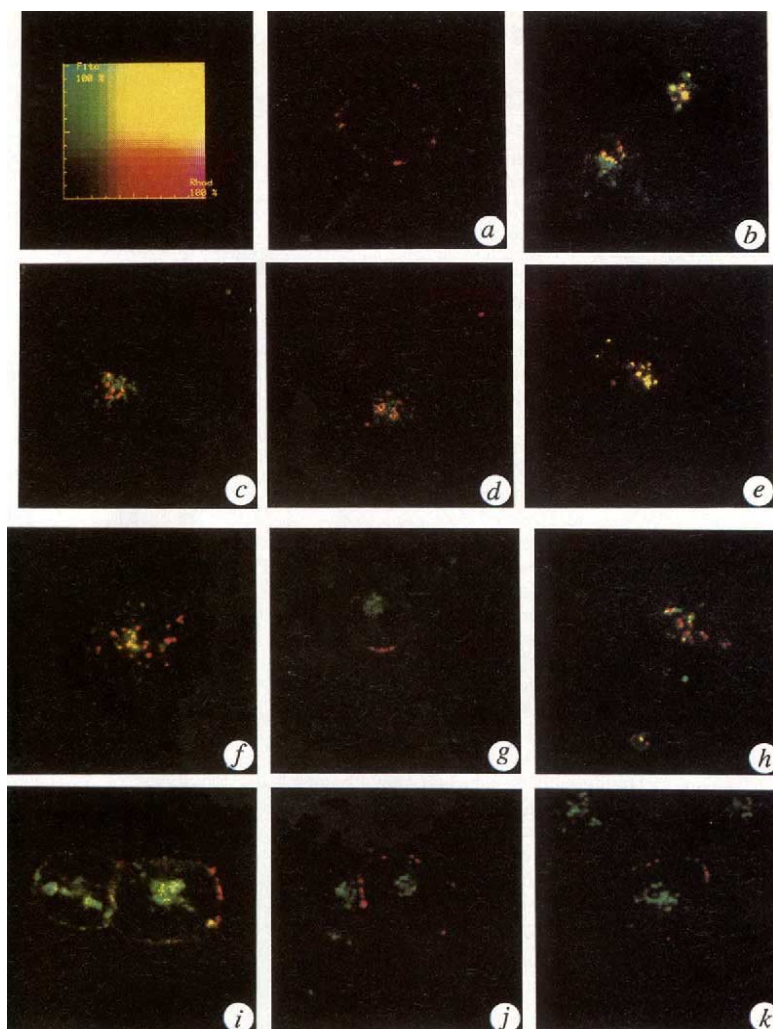


FIG. 1 Internalization and intracellular localization of Fc γ RIII and of chimaeric receptors. In the upper panels *a* to *e*, transfectant IIA1.6 cells expressing wild-type Fc γ RIII (α , γ 2) were pre-incubated with the monoclonal antibody (mAb) 2.4G2 and then with rhodamine-coupled mouse anti-rat IgG and FITC-coupled transferrin at 4 °C. Cells were then incubated at 37 °C for 0 min (*a*), 5 min (*b*), 10 min (*c*), 20 min (*d*). In *e*, FITC-coupled transferrin was replaced by FITC-coupled α_2 -macroglobulin (30 min). Slides were processed for fixation and mounting and analysed by confocal laser scanning microscopy. Representative fields of 35 $\times$ 35 μ m² among series of at least 8 specimens are shown. The pseudo-colour scale used to visualize the double-labelled confocal micrographs is depicted in the upper left panel (rhodamine signal in red, fluorescein signal in green, yellow colour indicates colocalization of both markers). In the lower panels *f* to *k*, Fc γ RIII (α ct4, γ 2; *f*), Fc γ RIII (α ct4; *g*), Fc γ RII chimaera (β /ct γ ; *h*), Fc γ RII tail-minus (β ct1; *i*) and mutated-Fc γ RII chimaeras (β /ct γ Y-V21, *j*; β /ct γ Y-L32, *k*) were crosslinked at 4 °C and then incubated for 20 min at 37 °C in the presence of FITC-coupled transferrin.

METHODS. B-cell transfectants were first allowed to adhere to a glass coverslip pre-coated with 0.2% poly-L-lysine and then incubated at 4 °C for 30 min with 10 μ g ml⁻¹ of 2.4G2, washed and incubated for another 30 min at 4 °C with rhodamine-coupled F(ab')₂ fragments of mouse anti-rat IgG. For transferrin uptake, transfectant cells prelabelled for Fc γ R were incubated with 60 nM of FITC-transferrin for 20 min at 37 °C, chilled, washed and fixed as described²². For kinetic studies, adherent cells were pre-incubated for 45 min at 37 °C in serum-free medium and further incubated with 2.4G2 at 4 °C. Cells were stained for 20 min at 4 °C with a solution containing both the rhodamine-coupled F(ab')₂ fragments of a mouse anti-rat IgG antiserum and the FITC-transferrin (60 nM). After incubation at 37 °C for the indicated times, cells were processed as described²² for fixation and mounting. Alternatively, FITC-coupled α_2 -macroglobulin (200 μ M) replaced the FITC-transferrin used in the kinetic experiments. Confocal laser scanning microscopy was done as described²². Representative medial sections of 8 horizontal slices separated by 0.5 μ m are shown.



Complementary DNAs encoding Fc γ RIII α - and γ -chains were co-transfected into antigen-presenting cells, the Fc γ R-defective B lymphoma cell line IIA1.6 (ref. 13), although B lymphocytes do not normally express this type of receptor. After introduction of a stop codon at position 5 (from the plasma membrane) of the α -chain cytoplasmic domain, we obtained surface expression of truncated α -chains in the presence (α ct4, γ 2) or absence (α ct4) of γ -chains (Table 1). Individual cell lines homogeneously expressing 1–3 $\times$ 10⁴ receptors per cell (Table 1; ref. 10) were isolated. We also constructed chimaeric molecules linking the extracellular and transmembrane domains of Fc γ RII to the cytoplasmic domain of the γ -chain (β /ct γ ; Table 1). Using site-directed mutagenesis, we replaced the tyrosines in positions 21 and 32 of the chimaeras into valine and leucine (β /ct γ Y-V21 and β /ct γ Y-L32), respectively (Table 1). These chimaeric molecules, as well as Fc γ RIIb2 and the tail-minus Fc γ RII (β /ct1), were also transfected into IIA1.6 cells and were expressed at 5–16 $\times$ 10⁴ receptors per cell (Table 1; ref. 10).

We first tested the effect of crosslinking on Fc γ RIII-mediated internalization. Using double immunofluorescence labelling, we determined the intracellular localization of an Fc γ RIII multivalent ligand, co-internalized with either transferrin or with α_2 -macroglobulin, as markers of early and late endosomes, respectively. Before internalization Fc γ RII-ligand and transferrin were located at the cell surface (Fig. 1*a*). After 5 min at 37 °C, both markers partially colocalized in the same intracellular compartments (Fig. 1*b*). Although transferrin began to recycle to the cell surface, after 10 to 20 min the two markers were detected in distinct vesicular structures (Fig. 1*c* and *d*),

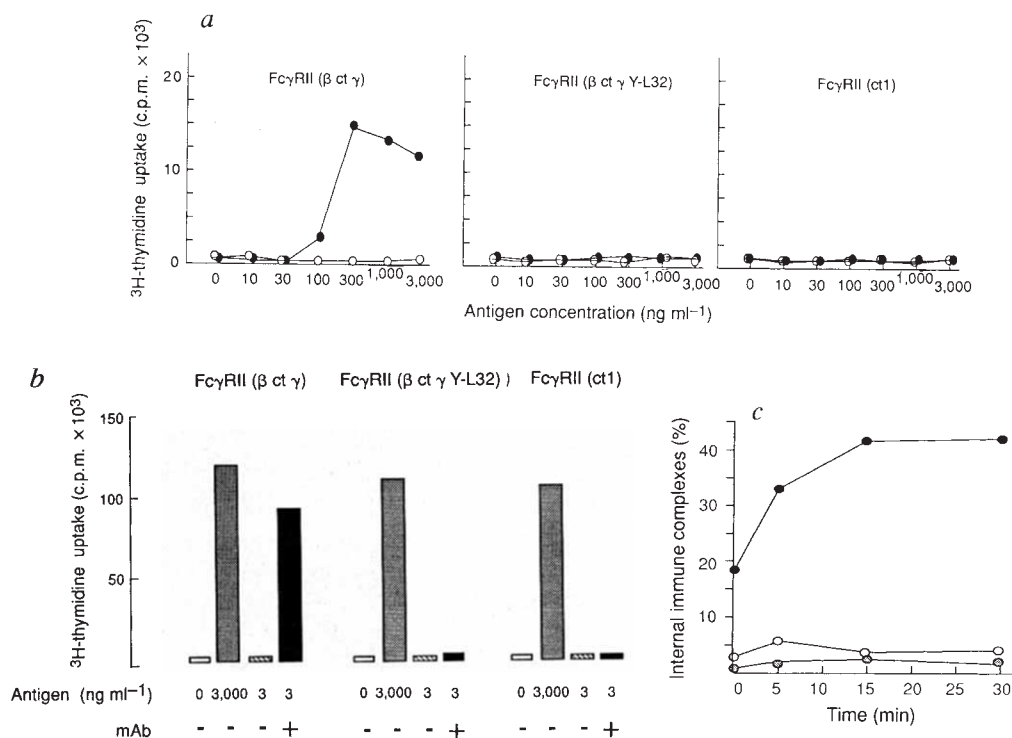
indicating that the crosslinked Fc γ RIII were no longer located in the early endosomes involved in transferring recycling. Conversely, when fluorescein isothiocyanate (FITC)-labelled transferrin was replaced by FITC- α_2 -macroglobulin, clear colocalization was detected with the ligands of Fc γ RIII after 30 min (Fig. 1*e*).

We next analysed the role of the associated γ -chain in internalization. Truncated or chimaeric Fc γ R were crosslinked and the cells incubated for 20 min at 37 °C in the presence of FITC-coupled transferrin to distinguish between surface-remaining and internalized complexes. In the presence of γ -chain, cytoplasmic domain-deleted Fc γ RIII (α ct4, γ 2) were efficiently internalized and were found in an endosomal compartment segregated from the early endosomes, as defined by a weak colocalization with transferrin (Fig. 1*f*). In contrast, the absence of γ -chain prevented ligand internalization via Fc γ RII (α ct4) (Fig. 1*g*). Therefore, the internalization process through Fc γ RIII is independent of the cytoplasmic domain of the ligand-binding α -chain, but requires the coexpression of γ -chains. Crosslinking of the chimaeric molecules (β /ct γ) triggered efficient endocytosis of the ligand and its accumulation in transferrin-negative endosomal compartments (Fig. 1*h*). In contrast, Fc γ RII minus tail (β ct1) mainly remained at the plasma membrane (Fig. 1*i*). Therefore, the cytoplasmic domain of the γ -chain contains a signal for receptor internalization. The mutation of any of the two tyrosine residues on the cytoplasmic tail of Fc γ RII chimaeric receptors (β /ct γ Y-V21 and β /ct γ Y-L32) prevented internalization after crosslinking (Fig. 1*j* and *k*). Thus, despite an unusually long distance between them (ten amino acid residues) for an endocytosis sequence^{14,15}, both

FIG. 3 Efficient presentation and internalization of IgG-complexed λ -repressor. *a*, λ -Repressor containing immune complexes induced the secretion of IL-2 by chimaeric receptor-expressing cells. Fc γ RII (β /ct γ), (β /ct γ -L32) or (ct1)-expressing cells were glutaraldehyde-fixed (circles) or not (filled circles) and incubated with immune complexes containing varying concentrations of λ -repressor. After 24 h, IL-2 release was tested. *b*, Fixation of the antigen-presenting cells after pulse incubation with antigen did not affect the presentation of low doses of IgG-complexed λ -repressor. Fc γ RII (β /ct γ), (β /ct γ -L32) or (ct1)-expressing cells were first incubated in the absence of antigen (open bars) or in the presence of either 3,000 ng ml⁻¹ (grey bars), 3 ng ml⁻¹ (hatched bars) or 3 ng ml⁻¹ complexed to 15 μ g ml⁻¹ of the two mAb anti- λ -repressor, 22D and 51F (solid bars). After extensive washing, cells were fixed with glutaraldehyde and recultured in the presence of the T-cell hybridoma 24.4. After 24 h, IL-2 release was tested. *c*, Internalization of prebound ¹²⁵I-labelled IgG-complexed λ -repressor by Fc γ RII (β /ct γ ; filled circles), (β /ct γ -L32; circles) or (ct1; grey circles)-expressing cells.

Cells were incubated with the labelled immune complexes for 30 min at 4 °C, washed extensively and transferred to 37 °C for the times indicated. Radioactive material remaining at the cell surface was eluted by acid to determine the amount of internalized material.

METHODS. *a*, Cells were activated by incubating 10⁵ cells in a final volume of 200 μ l in the presence immune complexes. IL-2 secretion was assessed as described. *b*, Presentation assays and fixation of the antigen-presenting



cells has been described²⁵. Briefly, after extensive washing cells were incubated in 0.05% glutaraldehyde (Sigma) for 30 s. Free sites were saturated by a 10-s incubation in 0.2 M glycine (Merk). *c*, Radioactive immune complexes were prepared with 3 μ g ml⁻¹ λ -repressor and 15 μ g ml⁻¹ mAbs 51F and 22D (labelled with ¹²⁵I using iodogen (Pierce)), centrifuged for 10 min at 10,000g to eliminate aggregates. Binding was measured after incubating cells for 30 min at 4 °C and acidic elution²⁶.

tyrosines influence the molecular structure responsible for internalization.

We then assessed the biological relevance of this internalization process, by investigating the ability of Fc γ RIII to increase the efficiency of antigen presentation to T lymphocytes, when antigen is complexed to IgG. As shown in Fig. 2a, untransfected IIA1.6 cells presented free λ -repressor or IgG-complexed only at high antigen concentrations (1,000–3,000 ng ml⁻¹), indicating that the binding of monoclonal antibody to the λ -repressor did not *per se* affect antigen presentation. In contrast, cells transfected with Fc γ RIII (α , γ 2), despite the low number of receptors expressed, presented IgG-complexed antigen at doses 30- to 100-fold lower than antigen alone. Interestingly, neither of the two antibodies alone mediated the increase of antigen presentation efficiency (data not shown), indicating that only multivalent ligands were efficiently internalized.

As for receptor internalization, the enhancement of antigen presentation of IgG-complexed λ -repressor via Fc γ RIII required the associated γ -chain and was independent of the cytoplasmic domain of the ligand-binding α -subunit (Fig. 2a). The chimaeric receptors (β /ct γ) presented IgG-complexed λ -repressor at concentrations three to four orders of magnitude lower than the doses required for antigen alone (Fig. 2b). The difference in the magnitude of the increase of antigen-presentation efficiency by the wild-type Fc γ RII (that is, 100) and the chimaeric receptors (that is, 3,000) may be accounted for by their respective levels of surface expression (Table 1). In contrast, with the tail-minus Fc γ RII (ct1)-expressing cells no significant increase in the antigen presentation efficiency was observed. Therefore the cytoplasmic domain of the γ -chain is required to increase the efficiency of presentation of IgG-complexed λ -repressor (Fig. 2b). The efficient presentation of IgG-

complexed λ -repressor involved Fc γ R because it could be blocked by the addition of the Fc γ R specific monoclonal antibody 24G.2 (refs 16, 17, and data not shown). Mutation of tyrosines 32 (Fig. 2b) and 21 (data not shown), prevented the efficient presentation of IgG-complexed λ -repressor by cells expressing the mutated chimaeras.

We next questioned the ability of the immune complexes used in the presentation assays to activate the transfected cells. As shown in Fig. 3a, λ -repressor complexed to IgG induced the release of interleukin-2 (IL-2) by antigen-presenting cells expressing the chimaeric receptors (β /ct γ) or wild-type Fc γ RIII (data not shown), but not the tail-less Fc γ RII (ct1). All the transfected cell lines were efficiently stimulated to secrete IL-2 via their B-cell antigen receptor (data not shown), as previously reported for these¹⁷ and other B lymphoma cells¹⁸. Similar results were found for protein tyrosine kinase activation and intracellular Ca²⁺ rise using these receptors¹⁰. In contrast, no effect of Fc γ R engagement on the level of expression of MHC class II molecules was observed (data not shown). Thus, as demonstrated for B-cell antigen receptor¹⁹ or Fc ϵ RI (ref. 20), Fc γ RIII mediates both ligand internalization and cell activation, which could be responsible for increasing the IL-2 levels found in antigen-presentation experiments. We therefore did antigen-presentation experiments using glutaraldehyde-fixed cells, which could no longer be activated by immune complexes to produce IL-2 (Fig. 3a). Glutaraldehyde-fixed cells expressing the chimaeric receptors (β /ct γ), but not the tail-less Fc γ RII (ct1) efficiently presented low antigen doses only when complexed to IgG (Fig. 3b). Furthermore, the presence of ovalbumin-containing immune complexes (which efficiently bind to FcR) did not affect the efficiency of presentation of uncomplexed λ -repressor by unfixed cells (data not shown). These results

exclude the possibility that the increased antigen presentation efficiency is due to IL-2 secretion by the presenting cells. Finally, we directly assessed the internalization of IgG-complexed λ -repressor, using 125 I-labelled 22D and 51F antibody. As shown in Fig. 3c, cells expressing the chimaeric receptors (β /ct γ), but not the tail-less Fc γ RII (ct1), efficiently and rapidly internalized pre-bound IgG-complexed λ -repressor. Together our data strongly suggest that the improvement of presentation obtained by complexing antigen to IgG is due to receptor-mediated internalization of the complexes.

Mutation of tyrosines 32 (β /ct γ Y-L32) or 21 (data not shown) in the chimaeric receptors prevented activation (Fig. 3a), efficient presentation by fixed cells (Fig. 3b), and internalization (Fig. 3c) of IgG-complexed λ -repressor. Although these two tyrosine residues are also involved in triggering activation of

protein tyrosine kinase of the *src* family¹⁰, the exact role of protein tyrosine kinase in Fc γ RIII-mediated endocytosis is unclear. But Fc γ RIII internalization was not only an overall consequence of cell activation, because crosslinking of surface IgG induced cell activation but not the internalization of pre-bound 125 I-labelled anti-Fc γ R antibody (data not shown).

In contrast to previously described internalization signals, which reside in the cytoplasmic domain of the ligand-binding polypeptides, Fc γ RIII-mediated endocytosis is determined by a structural motif containing two tyrosine residues, present in the cytoplasmic domain of the associated γ -chains. The presence of associated subunits structurally related to γ -chains in Fc ϵ RI, and in T- and B-cell antigen receptors suggests that associated chains may have a general role in the internalization of multimeric receptors. □

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Deformed autoregulatory element from *Drosophila* functions in a conserved manner in transgenic mice

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THE striking similarities in the structure, organization and anterior–posterior expression patterns between the murine Hox gene system and the *Drosophila* homeotic gene complexes^{1,2}, called HOM-C (ref. 3), may point to highly conserved mechanisms for specifying positional identities (reviewed in ref. 4). Strong support for this concept lies in the observation of conserved colinearity between the genomic order of the Hox/HOM genes and their unique successive expression domains along the anterior–posterior axes of both mouse and fly embryos^{1,2}. These unique and precise expression patterns appear to be facilitated by multiple *cis*-regulatory elements (reviewed in ref. 5). One of the few elements characterized in detail is the autoregulatory enhancer of the homeotic gene *Deformed* (*Dfd*)^{6–9}, which supports expression in subregions of posterior head segments of *Drosophila* embryos⁷. Here we present evidence that this enhancer is capable of conferring reporter gene expression to a discrete subregion of the hindbrain in transgenic mouse embryos. Remarkably, this anterior–posterior subregion lies within the common anterior expression domain of the *Dfd* cognate Hox genes in the postotic hindbrain^{10,11}. Our results indicate that the *Dfd* autoregulatory enhancer is part of a highly conserved mechanism for establishing region-specific gene expression along the anterior–posterior axis of the embryo.

To determine whether the *Dfd* autoregulatory enhancer from *Drosophila* can direct region-specific gene expression during mouse development, we prepared the reporter gene constructs

pLZ-Dfd/5C and pLZ-Dfd/7J containing the *Dfd* element in the normal or opposite orientation, respectively, in front of the murine *Hox-3.1* promoter region¹² and the *Escherichia coli lacZ* gene (Fig. 1). The *Hox-3.1* promoter region was used as a minimal promoter as it alone does not drive *lacZ* expression. This was established using the pLZ- Δ Dfd construct as a control (Fig. 1). None of the five independently produced transgenic embryos carrying this construct showed detectable β -galactosidase activity at 10.5–11.5 days of gestation (data not shown). This is consistent with reports indicating random or a total lack of reporter gene activity in transgenic mice carrying deletion constructs containing only proximal Hox gene promoter regions: that is, *Hox-1.3* (ref. 13), *Hox-2.6* (ref. 14) and *Hox-3.1* (C. J. Bieberich, personal communication). Ten transgenic mice were produced with the pLZ-Dfd/5C construct, of which four showed a discrete β -galactosidase expression pattern of variable intensity in the embryonic hindbrain at 10.5 days of gestation (Figs 1 and 2a). Essentially the same β -galactosidase pattern in the hindbrain was observed with six transgenic mice that carried the pLZ-Dfd/7J construct (Figs 1 and 2e).

Embryos of the permanent transgenic line 2S4 that carried the pLZ-Dfd/5C construct were among the strongest expressors of β -galactosidase; this was predominantly in a narrow ventral region of the hindbrain posterior to the otic vesicle (Fig. 2a, c). This spatially localized expression has a posterior limit at the level of the first cervical prevertebra and extends anteriorly to the level of the third branchial arch, which could be distinguished better in 2S4 embryos that were a few hours younger than 10.5 days post coitus (p.c.) (Fig. 2d). In the same region, we also observed β -galactosidase activity in cranial nerves X and XII, which were identified on the basis of their characteristic morphologies¹⁵ (Fig. 2a, c, d). Separated from this domain of reporter gene expression in the post-otic hindbrain, we noticed weaker expression in two parallel clusters of cells in the ventrolateral myelencephalon at the level of the otic vesicle (Fig. 2b, c). This seemed to be characteristic for 2S4 and was not found in the other transgenic mice, which showed overall weaker expression. In addition, *lacZ* expression was frequently detected in singular, irregular clusters of epidermal cells in the roof of

FIG. 2 Detection of *lacZ* reporter gene expression in whole-mount 10.5 days p.c. embryos. Transgenic embryos of the permanent lines 2S4 (a–d) and 3A5 (e) carrying either the pLZ-Dfd/5C or the pLZ-Dfd/7J construct (Fig. 1), respectively, were monitored for their β -galactosidase (β -gal) activity patterns, as indicated by the dark blue staining. β -gal activity is predominantly seen internally posterior to the otic vesicle, ov, within a narrowly restricted region of the head (a). A dorsal close-up view of that region in a second 2S4 embryo indicates β -gal activity located mainly in the two parallel lobes of the posterior myelencephalon; weaker activity can be detected separately from this region in two small parallel cell clusters of the myelencephalon at the level of the otic vesicle (b). A lateral close-up view of the cervical/posterior cranial region of the embryo in b indicates that β -gal activity in the postotic myelencephalon is ventrally restricted at the level of branchial arches b4 and b3 (c). In addition, β -gal activity is discernible in certain cranial nerves, n, presumably in nXII and nX, which emanate from this region^{15,17}. A lateral view of the same region from a 2S4 embryo a few hours younger than 10.5 days p.c. shows a more distinct anterior β -gal expression limit in the postotic hindbrain at the level of branchial arch b3 and comparably stronger β -gal activity in nerve nX (d). A close-up view of this region from a 10.5 days p.c. 3A5 embryo shows essentially the same spatial pattern of β -gal activity, although it is considerably weaker (e). b1 and b3, Branchial arches b1 and b3, respectively; C2, second cervical prevertebra; fb, forelimb bud; ms, mesencephalon.

METHODS. Embryos were dissected in the middle of the 10th day after either the day of vaginal plugging (permanent lines) or the evening of embryo reimplantation (transient). Embryos were fixed at room temperature in 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min, rinsed three times in PBS for a total of 20–30 min and incubated at 37 °C overnight in a solution containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) at 0.5 mg ml⁻¹, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂, 1 mM spermidine, 0.02% Nonidet P-40 (Sigma) and 0.01% sodium deoxycholate. After staining, embryos were postfixed in 3% paraformaldehyde in PBS at 4 °C for about 8 h, rinsed three times in PBS, and stored at 4 °C in 70% ethanol.

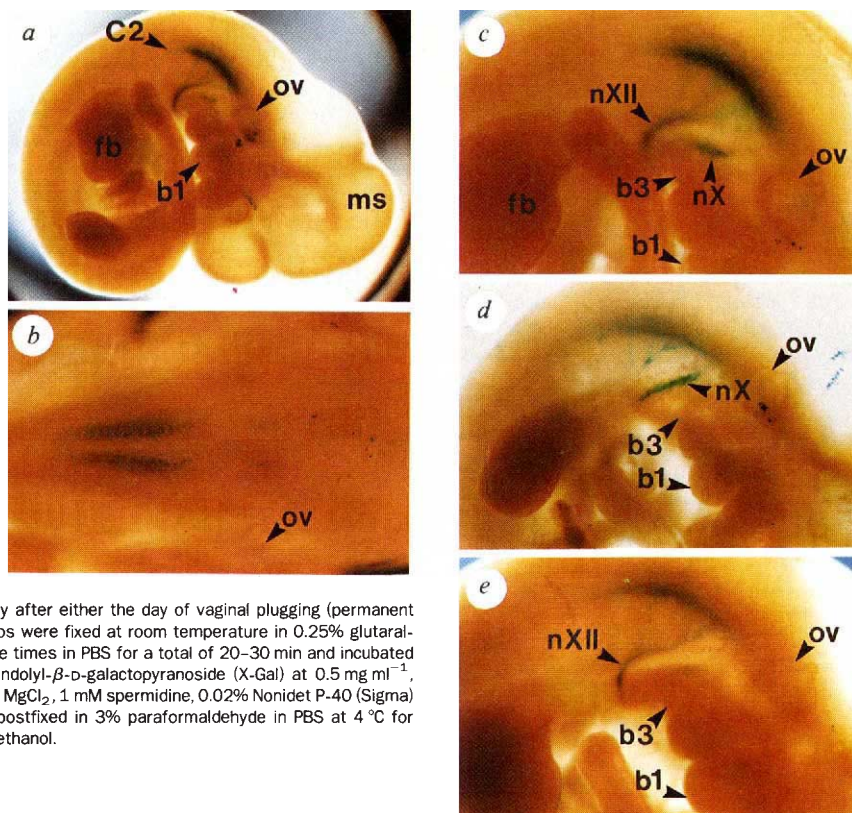
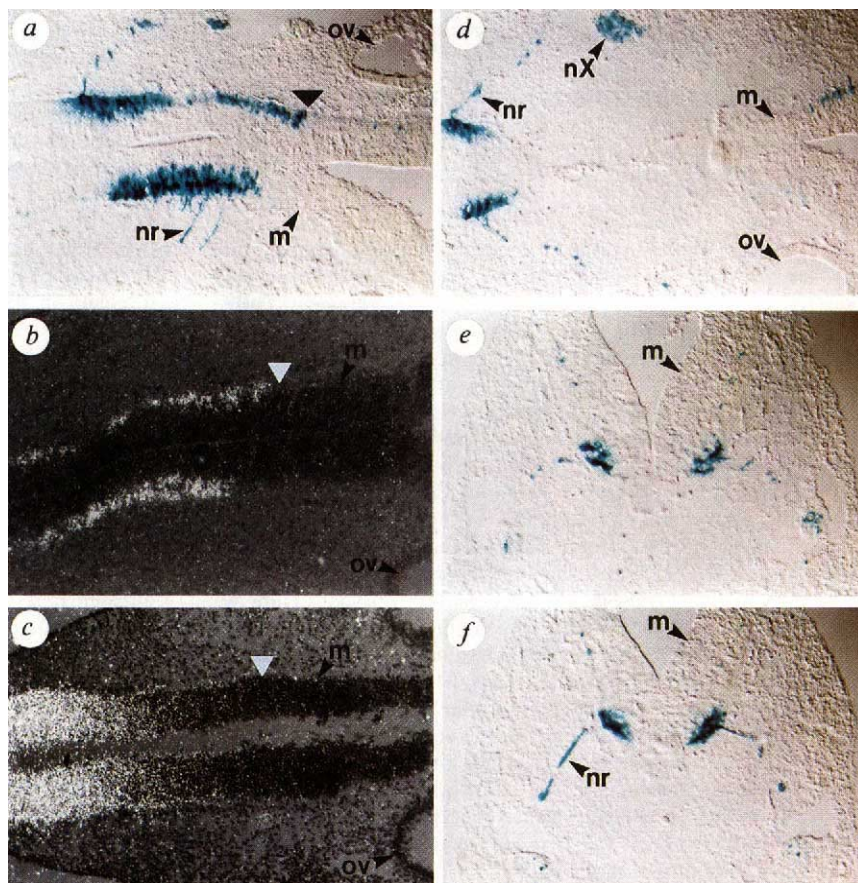


FIG. 3 Localization of *Dfd-lacZ* transgene expression in the hindbrain. Frozen horizontal sections (10 μ m thick) through the hindbrain region of X-Gal-stained 10.5 days p.c. 2S4 embryos show a discrete anterior–posterior domain of β -gal activity (blue stain) in the lateral myelencephalon, m, with a distinct anterior boundary (black triangle) just posterior to the otic vesicle, ov (a). A separate, smaller region of weak β -gal activity in the myelencephalon at the level of the otic vesicle is seen in a section from a deeper sectioning plane (d), which also shows β -gal activity in a cranial nerve, presumably nerve X, nX. Cross sections through the postotic myelencephalon close to the anterior boundary of strong β -gal activity (e) and more posteriorly (f) show that β -gal activity is predominantly restricted to the ventro-lateral myelencephalon, as well as to certain cranial nerve roots, nr, which are also seen in the horizontal sections (a, d). β -Gal activity is also detectable in a string of cells dorsal to the strongly stained ventro-lateral myelencephalon (e, f). *In situ* hybridizations to frozen horizontal sections (10 μ m thickness) through the hindbrain of a 10.5 days p.c. 2S4 embryo (b) and a non-transgenic littermate (c) with a probe specific for the *E. coli lacZ* (b) or the *Hox-1.4* gene (c) show the approximate anterior expression boundaries for both genes (white triangles) within the same region of the postotic myelencephalon. Note the restriction to the lateral myelencephalon of *Dfd-lacZ* transgene expression (b); *Hox-1.4* is expressed across the myelencephalon, with signal strengths decreasing to background towards its anterior expression boundary (c) (compare with the *Hox-1.4* expression pattern in the myelencephalon of 9.5 days p.c. embryos¹¹). *In situ* hybridization was as described³¹ using ³⁵S-labelled antisense RNA synthesized with SP6 polymerase from *Xho*I-linearized pLZRV-A (ref. 27) template DNA for the generation of *lacZ* gene-specific probe (2.4 $\times$ 10⁸ c.p.m. per μ g RNA) and from *Eco*RI-linearized plasmid p2181 B2 4a (ref. 32) for the generation of *Hox-1.4*-specific probe (2.9 $\times$ 10⁸ c.p.m. per μ g). After hybridization and washing, slides were coated with undiluted Kodak NTB photoemulsion and exposed at 4 °C for 10 days. Photographs were taken on a Zeiss Axiovert with DIC optics (a, d–f), or with darkfield illumination after counterstaining the sections with Giemsa (b, c).



the myelencephalon and anterior of the otic vesicle, as well as in clusters of head mesenchymal cells located dorsal of the eye and anterior of the otic vesicle (Fig. 2a, c, d). Six of the transgenic mice expressing the pLZ-Dfd/7J construct (Fig. 1) showed essentially the same β -galactosidase pattern in the postotic hindbrain as the 2S4 line, albeit at a weaker level (Fig. 2e). The ability of the *Dfd* autoregulatory element to function in either orientation has also been described in transgenic flies⁷.

A more detailed localization of the β -galactosidase expression pattern in 10.5 days p.c. 2S4 embryos is shown in horizontal and cross sections of the posterior hindbrain region (Fig. 3a, d–f). These indicate that β -galactosidase activity is restricted to distinct ventro-lateral subregions of the myelencephalon, which include cells of the marginal and mantle layers. In addition, expression is detectable in certain cranial nerve roots emanating from the stained region posterior to the otic vesicle (Fig. 3a, d–f), which we believe converge to cranial motor nerve XII on the basis of the β -galactosidase patterns seen in whole mount embryos (Fig. 2). The cluster of stained cells located laterally, posterior to the otic vesicle (Fig. 3d) corresponds presumably to cranial nerve X, also known as the vagus nerve. Nerves X and XII exit from neighbouring rhombomeres r7 and r8, respectively^{16,17}. It would be interesting to know how the restriction of the transgene expression seen in the cross-sections (Fig. 3e, f) compares to the dorso-ventral and medio-lateral patterns of the *Dfd* cognate Hox genes. *Hox-2.6* is known to be expressed across the entire postotic hindbrain at 10.5 days p.c. (ref. 18),

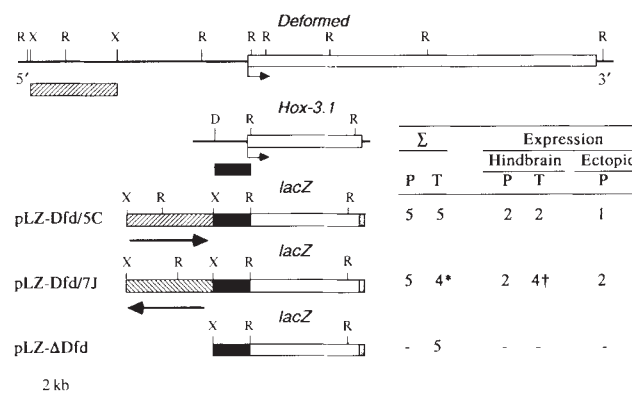
but no directly comparable data have been reported for the other three *Dfd* cognate genes (Fig. 4). Comparative studies with sagittal and horizontal sections from 12.5 (ref. 10) and 9.5 days p.c. embryos¹¹ respectively, however, suggest expression of *Hox-1.4*, -2.6, and -4.2 across the entire postotic hindbrain. Accordingly, the ventro-lateral restriction of the *Dfd* transgene expression may represent a subpattern of any of these cognate gene expression patterns. Embryonic stages later than 10.5–11.5 days p.c. were not analysed systematically, but a discrete pattern of β -galactosidase expression consistent with the pattern at the earlier stages persists at least until 13.5 days p.c., the latest stage tested. Most interestingly, β -galactosidase activity was detectable in proximal and distal regions of nerve XII, reaching to the level of the tongue of whole mount 2S4 embryos dissected after staining (data not shown).

Interestingly, in the hindbrain of 9.5 days p.c. embryos, the murine *Dfd* cognate genes *Hox-1.4*, -2.6 and -4.2 have a common anterior expression boundary at the rhombomeres r6/r7 junction¹¹. Rhombomeres are the repetitive segmental units of the vertebrate hindbrain (reviewed in ref. 19), which are particularly easily discernible at 9.5 days p.c. in the mouse. At 9.5 days of gestation no expression was detectable with the *Dfd*-constructs tested, so the anterior boundary of transcriptional *lacZ* gene expression in 2S4 embryos was compared with the *Hox-1.4* (ref. 20) boundary in the hindbrain of 10.5 days p.c. embryos (Fig. 3b, c). As expected, the *in situ* hybridization pattern in the postotic myelencephalon obtained with a *lacZ*

FIG. 1 Generation of *Dfd-lacZ* transgene constructs and transgenic mice. The top schematic shows the location of the 2.7-kb *XbaI* fragment (hatched box) containing the *Dfd* autoregulatory element about 4 kb upstream of the *Dfd* transcription unit^{7–9} (open box). The location of the 1-kb *DraI*-*EcoRI* fragment containing the *Hox-3.1* promoter (black box) in front of the *Hox-3.1* transcription unit¹² (open box) is shown next. The *lacZ* reporter gene constructs pLZ-Dfd/5C and pLZ-Dfd/7J were generated by cloning the *XbaI* fragment from the *Dfd* gene in both orientations into the cognate site in front of the *lacZ* reporter gene (open boxes) contained in the pLZRV-B plasmid. The blunt-ended *DraI*-*EcoRI* fragment containing the transcriptional start region of *Hox-3.1* isolated from mouse genomic clone cosMoEA (ref. 12) was inserted into the blunt-ended *Bam*HI site located between the *Dfd* fragment and the *lacZ* gene, and its original orientation was checked by nucleotide sequencing²⁶ using custom-made primers. Plasmid pLZRV-B is similar to the pLZRV-A plasmid²⁷, except that the *lacZ* gene was cloned in the opposite orientation. The pLZ- Δ Dfd construct was prepared by deleting the *XbaI* *Dfd* fragment and used as a control. The shaded boxes at the ends of the *lacZ* gene constructs represent the simian virus 40 polyadenylation signal. All *EcoRI* (R) restriction enzyme sites are shown, as well as the *XbaI* (X) and *DraI* (D) cloning sites. Gene constructs were separated from vector DNA by restriction endonuclease digests using the unique flanking *NotI* and *XhoI* sites, followed by sucrose gradient fractionation²⁸. Fractions containing the purified transgene fragments were pooled, dialysed against 10 mM Tris/0.25 mM EDTA, pH 7.5, and the DNA concentration adjusted to 3 μ g ml⁻¹ for the injection into (CD-1 $\times$ B6D2) F1 single-cell embryos as described^{29,30}. For each construct, the number of transgenic mice was determined by Southern blot analysis of genomic DNA prepared from tail biopsies of weaned mice or from placentas³⁰. The number of permanent transgenic lines (P, for permanent) and the number of injected embryos (T, for transient), which were shown to be transgene carriers, are listed under the heading Σ in the table on the right. *E. coli lacZ* gene expression was monitored (see Methods, Fig. 2 legend) in embryos of the F₁ or F₂ generation from permanent transgenic lines (P), and in injected embryos (T). The numbers of permanent lines and injected embryos showing the discrete neuronal expression pattern in the postotic hindbrain at 10.5 days of gestation (Fig. 2) are listed under 'Hindbrain'. The last column lists the numbers of transgenic mice (all permanent lines) giving unique *lacZ* expression patterns in distinct subregions of neuronal, mesodermal and epidermal tissue, but without the characteristic pattern in the postotic hindbrain. No *lacZ* expression was detectable in the remaining transgenic mice.

* The total number of injected embryos carrying this construct was not determined, because only those expressing *lacZ* at 10.5 days of gestation were analysed by Southern blotting.

† In addition to the characteristic hindbrain pattern, one injected embryo showed strong expression in separate distinct regions of the fore- and midbrain.



gene-specific probe (Fig. 3b) matches the β -galactosidase pattern (Fig. 3a), and the anterior expression limit in that region appears to be in close proximity to the *Hox-1.4* boundary (Fig. 3c). *Hox-1.4* expression in the postotic myelencephalon of 9.5 days p.c. embryos is known to decrease anteriorly until it reaches background levels at the r6/r7 boundary¹¹. Because of this, it is difficult to determine with certainty the anterior limit of *Hox-1.4* expression.

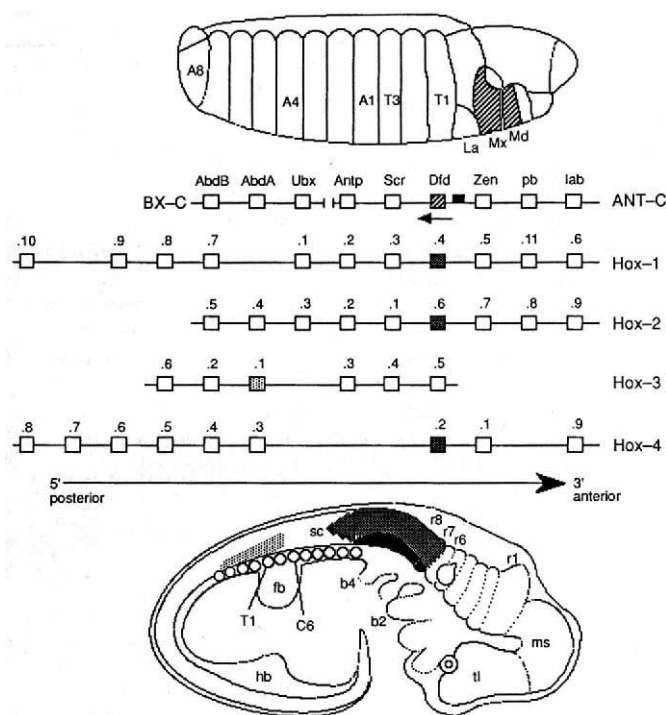


FIG. 4 Schematic representation of *Dfd/lacZ* transgene expression in relation to the *Dfd* cognate Hox genes. The approximate *Dfd* expression pattern (hatched) in subregions of the maxillary (Mx) and mandibular (Md) head segments of a *Drosophila* embryo at the retracted germ band stage⁸ is shown at the top. The labial head segment (La), the three thoracic (T1–T3), and the eight abdominal segments (A1–A8) are also indicated. The ANT-C and BX-C homeotic gene clusters from *Drosophila* are shown in alignment to the four Hox gene clusters. The relative position and orientation of the *Dfd* gene (hatched box and small left arrow) and the *Dfd* autoregulatory region (small black box) in the ANT-C complex are indicated, as well as the three *Dfd* cognate Hox genes *Hox-1.4*, *-2.6*, and *-4.2* (finely stippled boxes) whose expression patterns in the embryonic hindbrain have been characterized^{10,11}. The arrow to the right indicates the 5'→3' orientation of the genes except *Dfd*, as well as the colinear relation between the order of the Hox and homeotic genes in their respective clusters and their anterior limits of expression along the anterior–posterior body axis^{1,2}. At the bottom, the predominant anterior–posterior expression pattern of the *Dfd-lacZ* transgene in the postotic hindbrain (black region) is superimposed over the approximate pattern of *Hox-1.4*, *-2.6* and *-4.2* in the murine CNS at 9.5 days p.c. (ref. 11), including their common anterior expression limit at the rhombomeres r6/r7 junction just posterior to the otic vesicle (oval structure in the segmented hindbrain). The pattern shown does not account for subtle anterior–posterior and dorso–ventral differences in expression between the three cognate Hox genes in the hindbrain region and the extent of expression in the spinal cord. Note the restriction of transgene expression in the hindbrain at the level of branchial arches b3 and b4, which may serve as anatomical landmarks in the absence of morphologically discernible rhombomere boundaries at 10.5 days p.c., the gestational stage of the idealized schematic shown here. The *Hox-3.1* expression pattern (coarse stipple) is also shown in the spinal cord at 10.5 days p.c., with its anterior limit at the level of cervical prevertebrae C6/C7 (ref. 33 and refs therein). This indicates no overlap with the expression pattern of the transgene, which contains the *Hox-3.1* promoter region (Fig. 1) in addition to the *Dfd* autoregulatory element. hb, Hindlimb bud; fb, forelimb bud; ms, mesencephalon; sc, spinal cord; tl, telencephalon. This schematic has been in part adapted from refs 1, 2, 4 and 17.

Taken together, our results demonstrate that the *Dfd* autoregulatory enhancer provides spatio-temporally restricted gene expression in the central nervous system (CNS) of mid-gestation mouse embryos. The spatial coordinates of expression in the postotic myelencephalon define a subdomain of the common anterior expression domain of the three *Dfd* cognate genes *Hox-1.4*, *-2.6*, and *-4.2* in the posterior hindbrain^{10,11} (Fig. 4). Although rhombomere boundaries could not be determined directly at this relatively late embryonic stage of 10.5 days p.c., our combined data suggest that transgene expression is limited to the r7 and r8 rhombomeres in the postotic myelencephalon. This shows a striking correlation with the double-segment periodicity in the morphological properties of the rhombomeres¹⁷ and the consecutive two-segment intervals of the anterior expression limits of certain neighbouring Hox genes such as *Hox-2.1*, *-2.6*, *-2.7*, *-2.8* (ref. 21). Interestingly, many neighbouring homeotic genes from the fruit fly also show two-segment intervals or approximate double-segment restriction in their expression patterns (reviewed in ref. 22). The close positional match in expression between the *Dfd* transgene and the *Dfd* cognate Hox genes is consistent with results demonstrating that a posterior head region-specific enhancer of the human *Dfd* cognate gene *HOX-4B* (ref. 23) confers restricted reporter gene expression in transgenic flies to a subregion of the normal *Dfd* expression domain which depends on the presence of the *Dfd* protein²⁴. Combined, these data are evidence for the conservation of the *Dfd*-autoregulatory feed-back loop⁸ in the arthropod and vertebrate lineages that have been phylogenetically separated for an estimated 600 million years. Conservation of transcriptional control mechanisms is not limited to the Hox/HOM gene systems, for a tissue-specific regulatory element of the alcohol dehydrogenase gene has been shown to be conserved between *Drosophila* and man²⁵. □

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A human *HOX4B* regulatory element provides head-specific expression in *Drosophila* embryos

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LIKE other homeobox genes of the Antennapedia and bithorax complexes (collectively called the HOM complex^{1,2}), the *Drosophila Deformed* (*Dfd*) gene has structural homologues in the *Hox/HOX* complexes of mouse and humans, one of which is human *HOX4B* (refs 3, 4). Previous experiments indicated that *HOX4B* protein can specifically activate the expression of the endogenous *Dfd* transcription unit in *Drosophila* embryos and larvae⁵. We therefore asked whether *HOX4B* cis-regulatory elements could mimic the function of a *Dfd* autoregulatory element⁶⁻⁸ in *Drosophila* embryos. Here we show that a *HOX4B* upstream element can surprisingly provide expression in a posterior head segment of *Drosophila*. One possible mechanism for the axial position-specificity of the human element may involve the conservation of a *Dfd*-specific autoregulatory circuit in both arthropod and chordate lineages. This possibility is supported by the finding that a *Drosophila Dfd* autoregulatory element supplies spatially localized expression in the hindbrain of mouse embryos⁹.

A 2.8-kilobase (kb) *HincII* fragment of human *HOX4B* (previously known as *HH0.c13*, *Hox-5.1* or *Hox-4.2*) upstream DNA

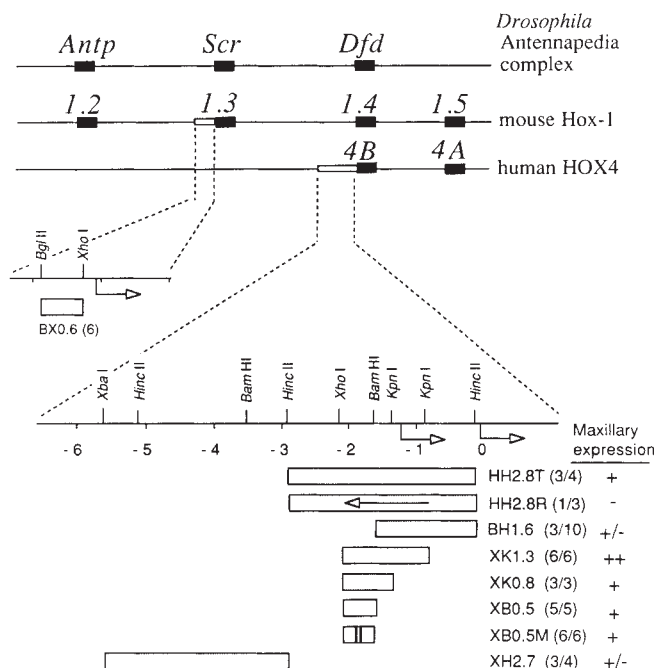
has been identified which directs expression of a reporter gene in the posterior hindbrain/cervical spinal cord of 10–14-day mouse embryos¹⁰. This expression pattern overlaps the anterior part of the normal central nervous system (CNS) expression pattern of *Hox-4.2* (the mouse homologue of *HOX4B*; refs 11–13). To test whether upstream sequences from *HOX4B* could provide spatially localized expression on the anterior-posterior axis of *Drosophila*, we cloned the 2.8-kb *HincII* fragment, as well as other fragments from the *HOX4B* 5' region, upstream of the *hsp70* promoter-*lacZ* reporter gene in the P-element transformation vector *HZ50* (ref. 14). Multiple *Drosophila* germ-line transformants were generated for each of the *HOX4B/HZ50* reporter constructs shown in Fig. 1, which included sequences that mapped from –5,700 to –60 base pairs (bp) relative to the *HOX4B* proximal cap site¹².

When attached to *hsp70-lacZ*, a tandem repeat of the *HOX4B* 2.8-kb *HincII* fragment, HH2.8T, activated *lacZ* expression in the maxillary segment during late stages of *Drosophila* embryogenesis. An adjoining 2.7-kb fragment from *HOX4B* upstream DNA, XH2.7 (Fig. 1), also directed maxillary-specific expression of *lacZ*, but gave less expression. The reversal of the 2.8-kb *HincII* fragment resulted in a fusion gene (HH2.8R) with nearly undetectable levels of activity, although still maxillary-specific (Fig. 1). Within the 2.8-kb *HincII* fragment, much of the maxillary regulatory function maps within the interval from –1.6 to –2.1 kb from the proximal cap site. Fragment BH1.6, which includes the interval from –1.6 kb to –60 bp, had virtually no activity in *Drosophila* embryos, whereas a fragment that spans the –2.1 to –0.8 kb region (XK1.3) had the highest activity tested (Fig. 1).

Expression of β -galactosidase from XK1.3 was first detected during embryonic stage 16, after maxillary segment cells have migrated to the anterior tip of the developing larva during the process of head involution (Fig. 2a). The XK1.3 expression pattern was restricted to 15–30 ventral-lateral cells in each of the paired maxillary lobes (Fig. 2b). These cells are included

FIG. 1 Mammalian regulatory elements tested in *Drosophila* embryos. At the top are shown a few of the *Drosophila* HOM genes in the Antennapedia complex vertically aligned with their mammalian homologues in the murine *Hox-1* and human *HOX4* complexes. Upstream regulatory elements (open bars) of human *HOX4B* and mouse *Hox-1.3* were fused to a basal promoter/*lacZ* reporter gene¹⁴ and used to generate germ-line transformants of *Drosophila*. Both mammalian regulatory elements are known to produce region-specific expression patterns in murine embryos¹⁰. *HOX4B* maxillary segment expression is dependent on the basal promoter sequences of *Drosophila hsp70* as well as *HOX4B* sequences, as the 2.8-kb *HincII* fragment attached to basal promoter and TATA sequences derived from mouse *Hox-1.3* (ref. 10) provides no embryonic expression, nor does the *hsp70* basal promoter in *HZ50* contain any inherent maxillary expression activity (ref. 14, and data not shown). Shaded bars below the restriction maps of the *Hox* upstream regions indicate the restriction fragments that were tested in transgenic *Drosophila*. The scale below the *HOX4B* restriction map measures the distance in kilobase pairs from the proximal transcriptional initiation site (arrow at 0). The distal transcriptional initiation site is also indicated by an arrow¹². The names given to particular *HOX4B* fragment/reporter constructs are indicated at lower right. The numbers in parentheses indicate the number of transgenic lines giving maxillary-specific expression/the total number of lines tested. Also indicated is an estimate of the relative regulatory strength of the fragments, based on the penetrance and relative staining intensity (calculated over all lines) of *lacZ* expression in stage-16 and -17 embryos (++ indicates >65% penetrance, strong staining; + indicates >15%–40%, strong staining; +/- is 5–15%, weak staining; minus means <1%, weak staining). Fragment HH2.8T was tested as a tandem dimer and the 604-bp fragment from the *Hox-1.3* locus was tested as a tetramer. Other fragments were tested as monomers. The two vertical bars in the XB0.5M fragment denote mutations in ATTA sites (see also Fig. 3).

METHODS. Fragments from the 5' regions of *HOX4B* and *Hox-1.3* were cloned between the *XbaI* and *KpnI* sites in the polylinker upstream of the *hsp70* promoter in *HZ50*. *HZ50* is a *ry+* P-element vector that contains *Drosophila hsp70* basal promoter sequences fused to a *lacZ* reporter gene¹⁴. *HZ50* constructs containing *Hox-1.3* or *HOX4B* fragments were



injected into the pole cell region of *ry⁻* embryos along with a helper plasmid p π 25.7wc (ref. 24), and transgenic flies were selected by eye colour²⁵. Mutation of the ATTA sites in the XB0.5 fragment was accomplished by polymerase chain reaction (PCR) overlap extension site-directed mutagenesis²⁶. The nucleotide sequence of the XB0.5M mutant fragment was confirmed by dideoxy chain-termination methods.

in the discrete expression pattern supplied by the *Dfd* epidermal autoregulatory element (Fig. 2a, b, e, f), and the XK1.3 pattern resembles the patterns given by some 'minimal' modules of the *Drosophila* autoregulatory element (refs 7, 8; and C. Zeng and W.M., unpublished results).

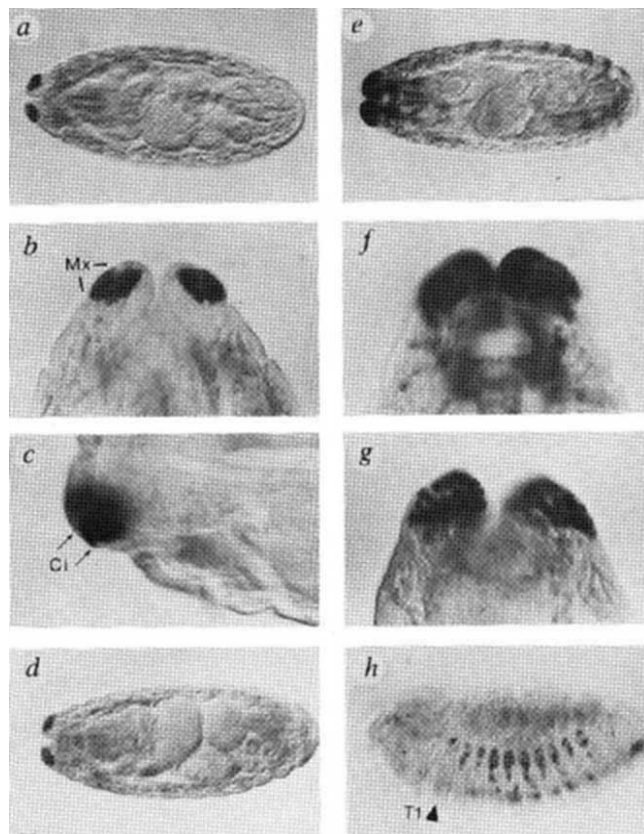
A *HOX4B/HZ50* fusion that contains only the -2.1 to -1.6 kb interval, XB0.5, also gave maxillary-specific expression, although at lower levels and penetrance than detected from XK1.3 (Fig. 2d). The sequence of the *HOX4B* XB0.5 fragment is remarkably similar to the mouse *Hox-4.2* upstream regulatory element (Fig. 3), which is activated in response to Hox-4.2 protein in embryonal carcinoma cells²⁹. The XB0.5 fragment has only two ATTA sites, commonly found as the core of HOM and Hox protein high-affinity binding sites¹⁵⁻¹⁹. Both of these sites match a high-affinity Dfd protein binding consensus (ATCATTAA; refs 8, 19) at 6 of 7 positions, and are imbedded in an extended region of nearly perfect sequence homology (133/140 base identities) between *HOX4B* and *Hox-4.2* (Fig. 3; and ref. 29). But these ATTA sites are not essential for the function of the XB0.5 fragment in *Drosophila*, because their mutation results in an element (XB0.5M) that still supplied maxillary-specific expression (Fig. 1).

Staining late-stage embryos with anti-Dfd antisera indicates that all cells that activate the *HOX4B* maxillary element also express Dfd protein (Fig. 2b, g). Thus it is possible that Dfd protein is directly involved in setting spatial limits on the expression pattern of the *HOX4B* element. In agreement with

this, *Dfd* null mutant embryos exhibit no *HOX4B* maxillary element expression (data not shown). But as the precise fate of all ventral maxillary cells in stage-16 embryos is unknown in *Dfd* null mutants, the lack of *HOX4B* element activity in such mutant animals may result from indirect causes. Although the human *HOX4B* maxillary element in the 0.5XB fragment shares functional similarities with attenuated versions of the upstream *Dfd* autoregulatory element, it shares no extensive primary sequence similarity with the 2.7-kb *Drosophila* element (ref. 8, and our unpublished results). This does not necessarily imply a lack of conserved functional elements as both *Drosophila* and human *Adh* upstream control elements conserve a functionally related region that has little primary sequence similarity, but still contains overlapping binding sites for the transcription factors AEF-1 and C/EBP (ref. 20).

To test whether a regulatory element from a more posteriorly expressed *Hox* gene would also direct maxillary-specific or other patterns of *HZ50* activation, we tested a mouse *Hox-1.3* upstream enhancer for activity in *Drosophila*. *Hox-1.3* protein sequence is most closely related to *Drosophila* Scr, but is also nearly as similar to the Antp and Ubx protein sequences^{2,21}. In mouse embryos, the 604-bp *Hox-1.3* upstream enhancer¹⁰ provides expression in the spinal cord, posterior to the domain of expression directed by the *HOX4B* element. In *Drosophila*, a tetramer repeat of the *Hox-1.3* enhancer (XB0.6) cloned into *HZ50* gave expression in a complex pattern in thoracic and abdominal cells of mid-to-late stage embryos, unlike the

FIG. 2 Expression patterns of *HOX* regulatory elements in *Drosophila* embryos. a, Ventral view of a late stage-16 *Drosophila* embryo transgenic for the *XK1.3/HZ50* fusion gene. β -Galactosidase expression directed by the human sequence is detected only in cells of the head region at late stages of embryonic development. Low levels of expression are also variably detected in the pharyngeal muscles, a pattern not dependent on *HOX4B* sequences as it is variably observed in all *HZ50* expression constructs. b, Close-up of the *XK1.3* expression pattern. High levels of β -galactosidase are present in ventral epidermis of the maxillary segment (Mx). c, Side view of the *XK1.3* expression pattern in a stage-17 embryo. β -Galactosidase expression is predominantly localized in the ventral row of cirri-producing cells. The apparent staining of the upper row is caused by signal outside the plane of focus. Arrows indicate the dorsal and ventral rows of cirri (Ci), specialized papillae that develop from ventral maxillary primordia^{27,28}. *Dfd* function is particularly important in cirri development, as *Dfd* mutants lack cirri, and ectopic expression of *Dfd* in embryos can induce the ectopic development of cirri in other head and thoracic segments². d, Ventral view of a stage-16 embryo transgenic for the *XB0.5/HZ50* fusion gene. As judged by staining intensity, β -galactosidase levels are reproducibly lower in XB0.5 than in *XK1.3* strains. In addition, the penetrance of the XB0.5 expression pattern is also lower than that of *XK1.3*. XB0.5 and *XK1.3* give expression in similar or identical sets of cells. e, A ventral view of a stage-16 embryo transgenic for an expression construct containing the 2.7-kb *Drosophila* sequence that comprises the *Dfd* epidermal autoregulatory element (*HZ2.7REV*; ref. 7). β -Galactosidase is expressed in both maxillary and mandibular epidermal cells, some of which have been incorporated into the anterior pharynx. f, Close-up of the embryo in e. The *HZ2.7REV* construct gives high levels of expression in ventral maxillary epidermis at this stage. g, Wild-type expression pattern of Dfd protein in an early stage-16 embryo. Most of the maxillary epidermal cells, particularly those on the ventral aspect, still accumulate Dfd protein at this stage. The punctate appearance of Dfd protein expression is due to its nuclear localization. Double stains with anti-Dfd and anti- β -galactosidase antiserum, differentially labelled by indirect immunofluorescence, indicate that all cells that stain for *XK1.3* also stain for Dfd protein (not shown). h, Lateral view of a *BX0.6/HZ50* embryo containing the *Hox-1.3* regulatory element shown in Fig. 1. Abundant β -galactosidase expression is observed in the lateral epidermis of thoracic and abdominal segments at stage 13. Expression is also detected in a few cells of each neuromere of the CNS from the extended germ-band stage to late stages of embryogenesis. This construct also provides low level global expression in most embryonic cells at late stages. Staining of maxillary segment cells above this 'background' level is rare, with a penetrance of ~1%. Two of three *Drosophila* control strains containing *HZ50* reporter genes exhibit staining in the head as well as in other embryonic regions (T. Haerry, personal communication). We have tested the expression patterns of these strains in antibody staining experiments done in parallel with *XK1.3*



and find that using conditions in which nearly all the *XK1.3* embryos exhibit obvious expression in the ventral maxillary epidermis, only a few of the *HZ50* embryos exhibit very weak expression in the head (and other) regions. The expression patterns in these control strains are not identical to each other, and in only one of the control strains does the head portion of the β -galactosidase expression pattern resemble the maxillary pattern in *XK1.3* embryos. T1 denotes the first thoracic segment. Q920-HZ embryos⁸ were used as a negative control in all antibody staining experiments. Collections and stainings of embryos have been described^{7,8}.

FIG. 3 *HOX4B* 'maxillary element' sequence compared to mouse *Hox-4.2* upstream sequence. The nucleotide sequence of the 518-bp human *Xhol*-*Bam*HI fragment (XB0.5 of Fig. 1) is designated 4B. This sequence spans the region from -2,109 to -1,592 relative to the human *HOX4B* proximal cap site (Fig. 1; ref. 12). Aligned with the human sequence is the nucleotide sequence (4.2) from a homologous region upstream of the mouse *Hox-4.2* transcription unit²⁹. Identities are indicated by dashes. Insertions in the mouse sequence relative to human are indicated by an inverted V underwritten by either the inserted base, or by the number of bases found inserted in the mouse sequence. Gaps in the mouse sequence relative to human are designated by gaps in the dashed mouse sequence. A 140-nucleotide region from -2,010 to -1,870 bp in the *HOX4B* sequence has only 7 base substitutions between mouse and human. Within this region are ATTA containing sites (shaded boxes) that contain 6/7 matches to a Dfd protein consensus ATCATTAA binding site (TTCATTA on the bottom strand of the 5' site, and ACCATTA on the top strand of the 3' site)^{8,19}. The base changes introduced into the ATTA sites in the XB0.5M construct are indicated in the shaded boxes.

	XhoI						-2050
4B	CTCGAGCCAG	GGCATCCCTG	TGCCCTTGAA	ATCCATCGAG	GCTCTGAGTG	AGTCTGCCGT	
4.2	-C-AG-----	-----C-	-----	--AGC-C-	C-C-A-----	CA---G---	
4B	CTCCATGCCC	TGAACTGAGG	GACAAGCACT	GCACAGTGCC	GAGGCGCTGG	GGCGGGTGAA	-1990
4.2	--TG-----	---T-----	-G-----C-A	-G-----C-		-----C---	
4B	ATTGTGTAAC	TTTTGTACTC	TTGTGTGCTG	CTGTCGGCAA	AGCAAAATA	ATGAAGTTT	-1930
4.2	---C-C-----	-----	--C-----	-----	-----	-----C---	
	NruI						
4B	GTGATATGTT	TGTAAATGAT	TTCGAATGAC	CCCTCGCCCT	GCCCTTACC	ATTAGCGCGC	-1870
4.2	-----	-----	-----	-----	-----	-----T-A-	
4B	CGGCCTCAGC	CCAAGCGCGC	CTCGGTGCCT	GTGAGCAGCG	CCTGCCTCGG	GGTCTGCAGC	-1812
4.2	-A-T-----	--- --GA-G	-----G-C	-CA-A-----	-----C-	-----	
4B	AGCAGCGGAA	CGGAGAGGCC	AGCCCGGATC	AGGGCCCATG	AAGGTAAATG	CGCGACTTCT	-1750
4.2	---TG--T	-C-A--T--	G--GG--C-G	-----GA	-----T	GT--A--	
	28 BamHI					C	
4B	TGGAGAAGTA	GCTTTGGCAG	ACAACCTGCG	TTGGTCGGGC	AGTAGGAAAA	GGAGTAGACA	-1690
4.2	A---G-C-	---G--A-CA	CTC--T----	--A-GTTCTG	GTG-A-TCT-	TTCCTTTTGC	
4B	CAGGATATTC	CCCTCTCGCT	TCTTTTCTTT	CTGCCGCTGA	GGGCATTGT	TCCTTTCTGG	-1630
4.2	-TTTT-CAAA	A-TC-CT	--	---T-----	---C-----	-T---T---	
				BamHI			-1592
4B	AAAAATACCAC	TTGGTAAGGA	GGAGGGTTAT	TTGGATCC			
4.2	G-----	---A-----G	--C--AG--	---G-T-			

HOX4B element pattern, and not obviously related to the expression pattern of any specific *Drosophila* HOM gene (Fig. 2h).

Curiously, most or all of the cells that activate the *HOX4B* element in *Drosophila* are epidermal, whereas the principal cells that activate the *HOX4B* element in mouse embryos are in the hindbrain/anterior spinal cord of the CNS. One way to reconcile this apparent paradox is to view these results as additional evidence for the idea that the fundamental patterning mechanisms underlying insect metameres and the vertebrate rhombomeres of the CNS are conserved^{22,23}. □

Faster superoxide dismutase mutants designed by enhancing electrostatic guidance

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THE enzyme Cu, Zn superoxide dismutase (SOD) protects against oxidative damage by dismuting the superoxide radical $O_2^{\cdot -}$ to molecular oxygen and hydrogen peroxide¹⁻³ at the active-site Cu ion^{4,5} in a reaction that is rate-limited by diffusion^{3,6} and enhanced by electrostatic guidance⁷⁻¹⁰. SOD has evolved to be one of the fastest enzymes known ($V_{\max} \sim 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$)^{6,11}. The new crystal structures of human SOD¹² show that amino-acid side chains that are implicated in electrostatic guidance⁸ (Glu 132, Glu 133 and Lys 136) form a hydrogen-bonding network. Here we show that site-specific mutants that increase local positive charge while maintaining this orienting network (Glu $\rightarrow$ Gln) have faster reaction rates and increased ionic-strength dependence, matching brownian dynamics simulations incorporating electrostatic terms. Increased positive charge alone is insufficient: one charge reversal (Glu $\rightarrow$ Lys) mutant is slower than the equivalent charge neutralization (Glu $\rightarrow$ Gln) mutant, showing that the newly introduced positive charge disrupts the orienting network. Thus, electrostatically facilitated diffusion rates can be increased by design, provided the detailed structural integrity of the active-site electrostatic network is maintained.

We used the tenfold redundancy of the new human SOD

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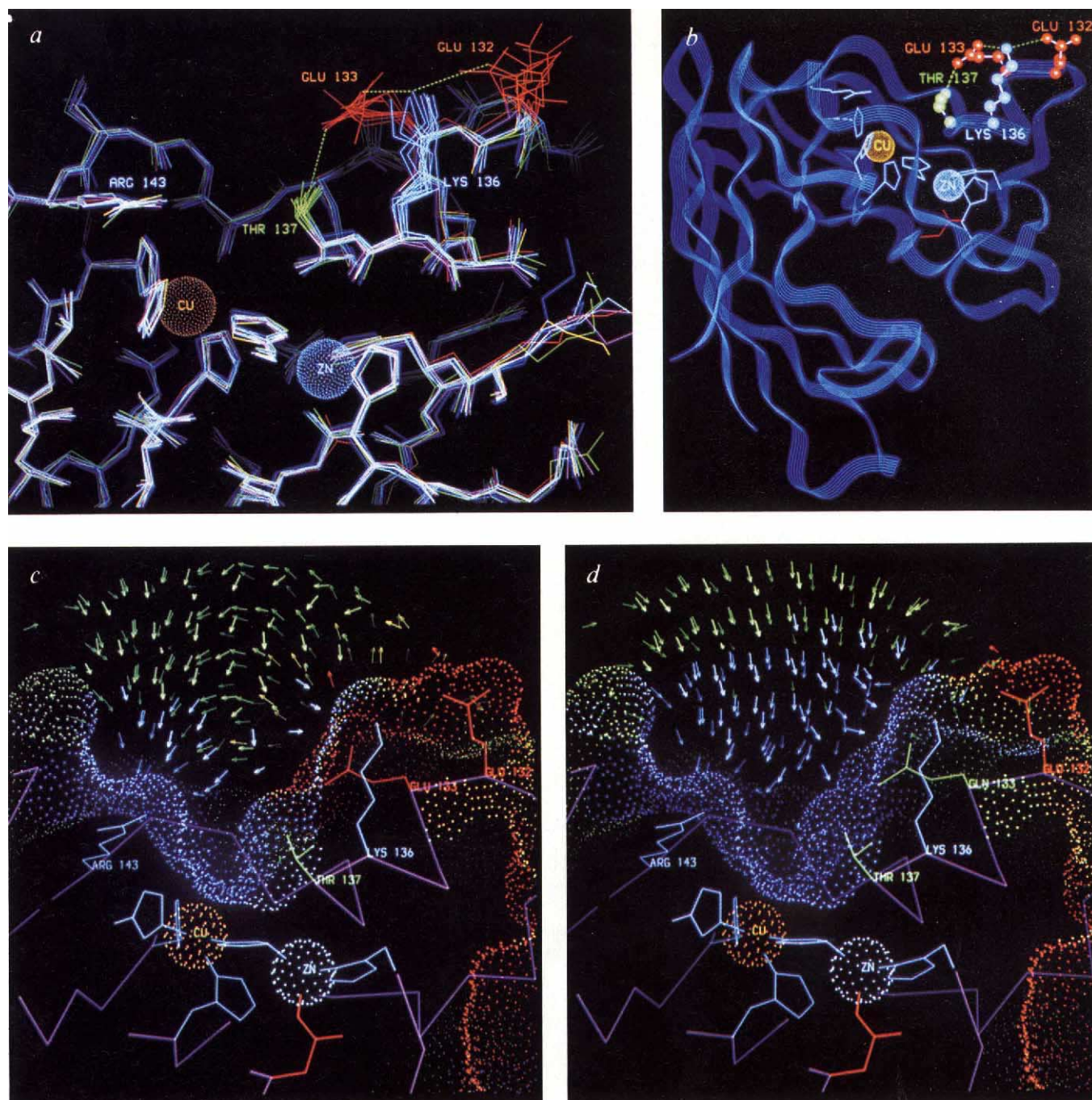


FIG. 1 Network of human SOD residues involved in electrostatic recognition of O_2^- substrate: Glu 132, Glu 133, Lys 136 and Thr 137. The electrostatic colour-code is: red, most negative; yellow, negative; green, neutral; light blue, positive; blue, most positive. Cu (gold) and Zn (blue) ions are shown as spheres. The SOD enzyme shown here, and used as the wild-type control for construction of our electrostatic mutants and for our rate measurements and calculations, is the double mutant Cys 6 → Ala and Cys 111 → Ser (refs 12, 20–22). This thermostable mutant maintains the activity and rate-versus-pH profile of wild-type human SOD²². *a*, All ten superimposed copies of the SOD active site. Yellow dotted lines (upper right) show hydrogen-bonds linking side chains of electrostatically important residues: Thr 137 (green, centre) to Glu 133 (red, top centre), to Lys 136 (blue, upper right), to Glu 132 (red, upper right). All other residues are shown colour-coded by subunit, and appear white where superimposed. *b*, Overview of mutated residues in the electrostatic loop and their structural relationship to the active site and overall β -barrel protein fold (blue ribbon following polypeptide chain) in one subunit of the SOD homodimer. Electrostatically important side chains Glu 132, Glu 133, Lys 136 and Thr 137 (coloured and oriented as in *a*) form a hydrogen-bonding network (ball-and-stick models with dashed hydrogen-bonds) on the solvent-exposed helical turn of the electrostatic loop overhanging the active-site channel (upper right). This helix is oriented so that its

C-terminal carbonyl oxygen atoms and helical dipole stabilize and are stabilized by Zn binding. Side chains of Cu and Zn ligands and of active-site Arg 143 are shown as lines. *c*, Cross-section of the active-site channel for wild-type human SOD, with electrostatically colour-coded molecular surface dots and arrows indicating the direction of the electrostatic force on negatively charged O_2^- . *d*, Corresponding view for (Glu 133 → Gln)SOD. Neutralizing negatively charged Glu (to isosteric Gln) increases the reaction rate by guiding O_2^- more directly downward to the catalytic Cu ion. This interpretation, based on a simple coulombic model, is consistent with the rate increases calculated with the Poisson-Boltzmann model used for the brownian dynamics simulations.

METHODS. For electrostatic calculations, partial charges²³ and radii²⁴ were assigned to atomic coordinates (including polar hydrogen atoms) for human (Cys 6 → Ala, Cys 111 → Ser)SOD (ref. 12), following guidelines used previously⁸. Electrostatic potentials and fields were calculated with the programs ESPOT and ESFIELD using a coulombic model with a distance-dependent dielectric value ($\epsilon = 1/r$, where r is the distance from the calculation point to the partial charge⁸). The electrostatic field or potential gradient was calculated by evaluating partial derivatives of the electrostatic potential along three coordinate axes. Field vectors (arrows) show the direction of maximum increase in electrostatic potential.

structures (five dimeric enzymes per asymmetric unit)¹² to define side-chain interactions of electrostatically important residues (Fig. 1a). Glu 132, Glu 133, Lys 136 and Thr 137 form a hydrogen-bonding network at the rim of the active-site channel, with the positively charged nitrogen of Lys 136 tethered between the negatively charged carboxylates of Glu 132 and Glu 133. Glu 133 lies closest to the substrate-binding pocket (located between Thr 137 and Arg 143) and orients the triad of charged residues by forming a charged hydrogen-bond to the hydroxyl group of Thr 137 (Fig. 1b). Structural interactions of electrostatically important side chains orient the electrostatic fields (Fig. 1c and d) and provide a basis for understanding the resultant rates of mutant enzymes.

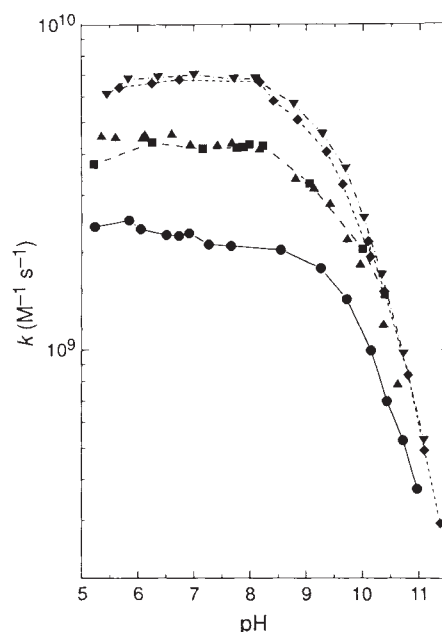
Single and double Glu→Gln mutants, which neutralize charges while minimally affecting size, shape and hydrogen-bonding properties, were prepared and their activities measured by pulse radiolysis (Fig. 2). In addition, (Glu 132→Gln, and Glu 133→Lys)SOD was made to test the prediction^{9,10} that reversing the charge on Glu 133 should increase the rate. Mutations neutralizing negative charges in the electrostatic loop lead to 2- to 3-fold rate increases. Glu 133 is more dominant than Glu 132 in determining reaction rate, consistent with its proximity to the O₂⁻ binding pocket (Fig. 1). Metal-site geometry is unperturbed in these mutants as shown by electron paramagnetic resonance (EPR) and ¹H NMR experiments on Cu, Co enzyme derivatives (L.B. *et al.*, manuscript in preparation). The more positively charged (Glu 132→Gln, Glu 133→Lys)SOD is significantly slower than (Glu 132→Gln, Glu 133→Gln)SOD (Fig. 2), indicating that factors besides charge influence the rate.

FIG. 2 Rate constants versus pH for wild-type and mutant human SODs as measured by pulse radiolysis. Double mutant (Glu 132→Gln, Glu 133→Gln)SOD (▼, dot-dashed line) and single mutant (Glu 133→Gln)SOD (◆, short-dashed line) have the highest rates, double mutant (Glu 132→Gln, Glu 133→Lys)SOD (■, long-dashed line) and single mutant (Glu 132→Gln)SOD (▲, dotted line) have intermediate rates, and wild-type SOD (●, solid line) has the lowest rate, at 0.01 M ionic strength. The pattern of constant activity from pH 5–8 for the Glu→Gln mutant enzymes resembles that of wild-type SOD.

METHODS. Rate data were obtained from pulse radiolysis experiments (6 measurements per data point; error <5%) with the 2-MeV van der Graaff accelerator at Brookhaven National Laboratory. Superoxide radicals were generated in air-saturated aqueous solutions²⁵ containing 10 mM sodium formate and 0.5 mM HEPES. Decay of O₂⁻ was monitored spectrophotometrically at 245–270 nm in the presence of 36–72 μ M EDTA, 1–10 μ M O₂⁻ and 1–10 μ M SOD. Catalytic rates were calculated using active enzyme concentrations determined by measuring the concentration of the activity-essential Cu ion by atomic absorption spectroscopy (in triplicate on a Pye-Unicam or GBC instrument, with an error <5% for Cu and Zn). Our measured rates for wild-type SOD match published values^{11,22}, showing that our recombinant enzyme is fully active. Multiple rate measurements in separate experiments as a function of pH or ionic strength gave consistent results, and showed all four mutants to be significantly more active than wild-type SOD. The wild-type SOD crystallographic structure¹² shows a single specific Cu-binding site located at each active site. NMR and EPR studies on the four mutants confirm that this is a single Cu site and indicate that the metal site geometry is unperturbed (L.B. *et al.*, manuscript in preparation). Pulse radiolysis data obtained with and without the chelator EDTA gave identical rates, showing that there were no free Cu ions to contribute to activity. Rate measurements for (Glu 132→Gln, Glu 133→Lys)SOD are less accurate because, with this mutant only, EDTA competed for Cu ion binding; measurements at varying EDTA concentrations indicated that rates for this mutant are accurate only to within 30%. Proteins were purified to ~95% homogeneity after periplasmic expression in *Escherichia coli* harbouring the plasmid pPHSOD1LacI⁹. The plasmid pPHSOD1LacI⁹ (to be published elsewhere) contains a synthetic human SOD gene²⁰ joined to the leader sequence of *Photobacterium leiognathi* Cu, Zn SOD²⁶ and the *lacI*⁹ gene²⁷ in a pBR322 derivative containing the *tacI* promoter²⁸. Mutants were made by substituting double-stranded oligonucleotides between unique *SpeI* and *Bam*HI sites of pPHSOD1LacI⁹. Processing occurred correctly using the *P. leiognathi* signal peptide to leave an N-terminal alanine. The synthetic SOD gene already incorporates the Cys 6→Ala and Cys 111→Ser thermostable mutations^{20,21}. This thermostable mutant^{12,20–22}, which maintains the activity and rate-versus-pH profile of wild-type human SOD, is the base from

Repulsion between Lys 133 and the structurally adjacent Lys 136 may disrupt the hydrogen-bonding network, conformation and charge distribution above the active-site channel. NMR studies for this mutant detect one binding constant for azide (L.B. *et al.*, manuscript in preparation), which is suggestive of local changes in the positioning of charges, rather than local accumulation of anions at a second nearby binding site.

Our results provide an overall measure of electrostatic recognition. At physiological pH, the net negative charge on the enzyme suggests that there is an electrostatic barrier to incoming O₂⁻ which should decrease with increasing ionic strength, thus accelerating the reaction. Instead, local electrostatic attraction is dominant, so raising the ionic strength decreases the reaction rate for both human SOD (Fig. 3) and bovine SOD^{13,14}. The slope of rate as a function of ionic strength (Fig. 3) measures the product of interacting charges^{14,15} of enzyme and approaching substrate (-1). Although Glu→Gln mutants are more active than wild-type enzyme, their activity decreases more steeply with increasing ionic strength (Fig. 3). Thus, an increased electrostatic attraction of substrate resulting from fewer negative charges above the active-site channel is damped more effectively by added ions. As with absolute rates, slopes of ionic-strength dependence (Fig. 3), and thus apparent charge on human SOD seen by the O₂⁻ substrate, are largest for (Glu 132→Gln, Glu 133→Gln)SOD (apparent charge about +1.8), lowest for wild-type SOD (+1.1), and intermediate for the single mutants (Glu 132→Gln)SOD and (Glu 133→Gln)SOD (+1.4 each), suggesting that Glu 132 and Glu 133 each reduce the apparent active-site charge by about -0.4. But the ionic strength



which our electrostatic mutants were derived and is the wild-type control for our rate measurements and calculations. Each mutant gene was sequenced in its entirety to confirm the correct sequence. Recombinant human SOD proteins were produced in *E. coli* MC1061 (ref. 29) at about 10% of total cellular protein after overnight induction of the *tacI* promoter with 0.2 mM isopropyl β -D-thiogalactopyranoside in 10 l of L-broth containing 100 μ g ml⁻¹ ampicillin and 0.1 mM CuSO₄ (ref. 28). Periplasmic proteins were released by osmotic shock³⁰ in 100 ml water containing 0.1 mM CuSO₄. Human SOD proteins were purified to ~95% homogeneity by heating at 65 °C for 2 h, followed by DEAE-Sepharose chromatography³¹. (Glu 132→Gln, Glu 133→Lys)SOD was prepared in two batches: one with and one without the heat step. No differences were noted between the two subsequent sets of rate measurements. All proteins were dialysed against 20 mM Tris buffer, pH 8, 1 mM CuSO₄, and then extensively against water. Protein concentrations were determined spectrophotometrically at 265 nm and by dry weight measurements³¹.

dependence of the double mutant with a reversed charge (Glu 132→Gln, Glu 133→Lys) matches that of the double mutant with a neutralized charge (Glu 132→Gln, Glu 133→Gln), indicating that added positive charge (Glu 133→Lys) does not enhance substrate attraction.

Simulations of more than 300,000 trajectories of incoming O_2^- substrates under the influence of brownian motion and the enzyme's electrostatic field reproduce the experimentally measured ionic-strength dependence of the wild-type and mutant enzymes to within 1–11% (average 5%) of the experimentally determined slopes (Fig. 3). Although calculated rates are overestimated with current parameters, as seen for previous calculations on bovine SOD^{9,10,16}, our calculations successfully distinguish single changes in charge between the wild-type and mutant enzymes, and correctly rank all but one of the rates. Calculated rates match experimental rates best (about twofold difference) for wild-type human SOD and the single mutants (Fig. 3a). The largest difference, about sixfold for (Glu 132→Gln, Glu 133→Lys)SOD (Fig. 3b), is probably due to structural changes resulting from electrostatic repulsions between mutated Lys 133 and the neighbouring Lys 136.

Evolutionary selection for conserving Glu 133 (ref. 17), des-

pite the higher rate of catalysis of (Glu 133→Gln)SOD, may arise because neutralization of Glu 133 increases inhibition by phosphate anions (our unpublished results). Structural conservation of charged side-chain hydrogen-bonds from Thr 137 and Lys 136 to Glu 133 in all ten subunits of human SOD (Fig. 1a), despite high solvent exposure, suggests a supporting function for uncharged Thr 137 (invariant in eukaryotes¹⁷) in positioning both negatively charged Glu 133 and positively charged Lys 136 side chains. Mutations of Thr 137 produce rate decreases of up to 30%¹⁸. A simple bond rotation of Thr 137 would reposition its hydroxyl group towards incoming O_2^- , as seen in molecular dynamics simulations¹⁹, releasing the Glu 133–Lys 136 ion pair and reducing unfavourable interactions between Glu 133 and O_2^- . This would provide a mechanism for both conformational stability and inducible complementarity in enzyme–substrate recognition.

Based on structural analysis and electrostatic calculations, we have designed, constructed and experimentally tested human SOD mutants that are significantly more active than the diffusion-limited wild-type enzyme. Our results demonstrate that computational techniques allow successful design of mutants made faster by improved electrostatic guidance, provided the

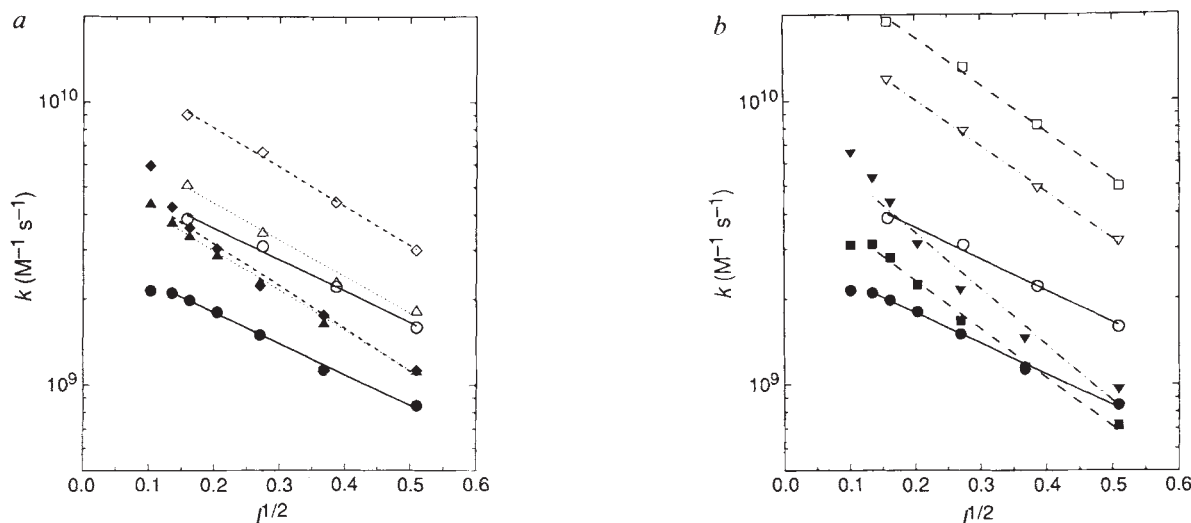


FIG. 3 Effect of ionic strength (I) on reaction rate ($\log k$ versus $I^{1/2}$ plots), as measured experimentally (solid symbols) by pulse radiolysis at pH 8.0 in HEPES with varying concentrations of NaCl, and calculated (open symbols) with brownian dynamics simulations (12,000 or more trajectories per data point). a, Rate data for wild-type SOD (circles, solid lines) and single mutants (Glu 132→Gln)SOD (triangles, dotted lines) and (Glu 133→Gln)SOD (diamonds, short-dashed lines). b, Rate data for wild-type (circles, solid lines) and double mutants (Glu 132→Gln, Glu 133→Gln) (inverted triangles, dot-dashed lines) and (Glu 132→Gln, Glu 133→Lys) (squares, long-dashed lines). $I = \frac{1}{2} \sum m_i z_i^2$, where m_i are molar concentrations of Na^+ , Cl^- and formate ions, and z_i are charges on these ions. Physiological ionic strength is about 0.15 M ($I^{1/2} \approx 0.4$). The slope ($\log k/I^{1/2}$) at low ionic strength ($I < 0.2$ M, $I^{1/2} < 0.45$) provides an approximation for the product of the interacting charges of substrate ($Z_{O_2^-} = -1$) and enzyme (Z_{SOD}) ions according to the equation^{14,15} $\log k = Z_{O_2^-} \times Z_{SOD} \times I^{1/2} + \text{constant}$. Solving for Z_{SOD} then provides a measurement of apparent charge of SOD as seen by incoming substrate O_2^- . On these plots, rate data at the lowest ionic strength ($I \approx 0.01$ M, $I^{1/2} \approx 0.1$) is comparable to rate data at pH 8 from pH profiles in Fig. 2 (same symbols).

METHODS. Brownian dynamics simulations were done with a version of the University of Houston Brownian Dynamics Program, developed by J. D. Madura and M. E. Davis in collaboration with J. A. McCammon, which we have modified. Partial charges and radii were assigned to the atomic coordinates (including polar hydrogen atoms) for human (Cys 6→Ala, Cys 111→Ser)SOD¹² as described in Fig. 1 legend. Electrostatic forces were calculated with the Poisson–Boltzmann method using a dielectric constant (ϵ) of 2 within the protein, and ϵ of 78 outside the protein, and applying dielectric boundary smoothing³². Molecular movements were modelled with hydrodynamic radii

of 2.05 Å and 28.5 Å for O_2^- and human SOD, respectively¹⁰. The electrostatic potential of human SOD was calculated onto a $128 \times 128 \times 128$ Å grid with 1.0-Å grid spacings centered around the SOD dimer. Distant electrostatic forces outside grid boundaries were calculated based upon a Debye–Hückel sphere of radius 30 Å and the net charge of each enzyme. Net charges of SOD dimers are -4 for wild type, -2 for single Glu→Gln mutants, zero for (Glu 132→Gln, Glu 133→Gln)SOD, and $+2$ for (Glu 132→Gln, Glu 133→Lys)SOD. Trajectories of substrate molecules approximated by spheres were started 41.5 Å from the centre of the dimer and pursued until reaction or exit from the system (500 Å radius sphere). Approach of O_2^- within 7.0 Å of either Cu ion constituted a reaction; this distance, matching the entrance of the O_2^- binding pocket, overcame numerical limitations arising from the size of grid spacings relative to van der Waals radii. Collisions of the centre of the O_2^- with the protein were detected using an exclusion grid calculated with the van der Waals radii of the protein atoms increased by 1.5 Å to include the radius of a superoxide's oxygen atoms. Timesteps for simulations increased with distance from the dimer centre: 0.10 ps within 40 Å, 0.25 ps between 40 and 100 Å, and 1.0 ps beyond 100 Å. Differences between our simulations and those done previously^{9,10,16} include: a different system, using new human¹² versus bovine SOD coordinates^{4,5}; different parameters, including definitions of target sites for reaction, partial charge assignments, a bigger grid and/or finer grid spacings; and changes in software, using a new version of the program, with our modifications to atomic radii defining the solvent/protein dielectric boundary and the collision boundary with the protein (not explicitly specified in previous publications), all of which probably contribute to subtle differences in results.

structural arrangement of important charged residues is maintained. □

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Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21

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THE stimulation of a variety of cell surface receptors promotes the accumulation of the active, GTP-bound form of Ras proteins in cells^{1–4}. This is a critical step in signal transduction because inhibition of Ras activation by anti-Ras antibodies or dominant inhibitory Ras mutants blocks many of the effects of these receptors on cellular function^{5–8}. To reach the active GTP-bound state, Ras proteins must first release bound GDP. This rate-limiting step in GTP binding is thought to be catalysed by a guanine-nucleotide-releasing factor (GRF)^{9–11}. Here we report the cloning of complementary DNAs from a rat brain library that encode a ~140K GRF for Ras p21 (p140^{Ras-GRF}). Its carboxy-terminal region is similar to that of CDC25, a GRF for *Saccharomyces cerevisiae* RAS¹¹. This portion of Ras-GRF accelerated the release of GDP from RasH and RasN p21 *in vitro*, but not from the related RalA, or CDC42Hs GTP-binding proteins. A region in the amino-terminal end of Ras-GRF is similar to both the human breakpoint cluster protein, Bcr¹², and the *dbl* oncogene product¹³, a guanine-nucleotide-releasing factor for CDC42Hs¹⁴. An understanding of Ras-GRF function will enhance our knowledge of the many signal transduction pathways mediated by Ras proteins.

To clone a GRF for Ras p21, (Ras-GRF), we exploited the fact that GRFs for yeast RAS can activate mammalian Ras^{11,15}. A set of degenerate oligonucleotides was designed to code for all peptides present in two highly conserved regions of three GRFs for yeast RAS, CDC25 (ref. 16) and SDC25 from *S. cerevisiae*¹⁷ and Ste6 from *Schizosaccharomyces pombe*¹⁸. The oligonucleotides were used in a polymerase chain reaction (PCR) with DNA from a rat brain cDNA library as template.

The 159-base-pair PCR product was then used to isolate multiple overlapping cDNA clones totalling ~5 kilobases (kb). These sequences contained an uninterrupted open reading frame that coded for a protein containing 1,244 amino acids and a predicted M_r of 142,654 (Fig. 1).

The sequence of 310 amino acids in the C-terminus of Ras-GRF was similar to a comparable region of CDC25 (28% identity), SOS, a putative Ras-GRF of *Drosophila melanogaster*³² (30% identity) (Fig. 2a), SDC25 and *S. pombe* Ste6 (sequences not shown). Because this region of the yeast proteins is sufficient to promote guanine nucleotide exchange by Ras proteins, the terminal 476-amino-acid region of Ras-GRF (p55^{Ras-GRF}) was expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase (GST). After affinity purification with glutathione agarose beads, the fusion protein was tested for activity on recombinant RasH p21. Figure 3a shows that GST-p55^{Ras-GRF} markedly enhanced the dissociation of [³H]GDP bound to Ras p21, when compared with GST or plain beads. Under the same conditions, GST-p55^{Ras-GRF} promoted the release of [³²P]GTP from Ras p21, but to a lesser extent (Fig. 3b).

The rate of [³²P]GTP binding to RasH p21 was also increased by GST-p55^{Ras-GRF} (Fig. 3c). This was expected, because the release of prebound GDP is the rate-limiting step in GTP binding¹⁹. A similar effect was observed with RasN p21. Under these conditions, there was no effect on one of the closest relatives of Ras, the RalA protein (55% identical)²⁰ or on the more distant CDC42Hs protein (24% identity) (Fig. 3c)²¹, suggesting that this domain of Ras-GRF is highly specific.

We also noted similarity between a section of 223 amino acids in the amino end of Ras-GRF (amino acids 238–461) and a region of the protein encoded by the breakpoint cluster region gene, *bcr* (Fig. 2b) (495–750) (28% identity and 46% similar if conservative changes are included)¹². This gene is located on human chromosome 22 and is the site of breakpoints that contribute to translocations found in chronic myelogenous leukaemia and acute lymphocytic leukaemia. The function of the Bcr protein in cells is poorly understood, although it has at least two biochemical activities. The N terminus contains a serine/threonine kinase²² and the C terminus contains a GTPase-activating protein for the Ras-related Rac protein²³.

But it is the section between these two domains that shows sequence similarity to Ras-GRF. The *dbl* oncogene product also contains a similar stretch of amino acids that is required for its transforming activity (Fig. 3)²⁴. Dbl has recently been shown to be a guanine nucleotide-releasing factor for the CDC42Hs protein¹⁴, suggesting that the comparable domains in Bcr and

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Ras-GRF are releasing factors for CDC42Hs or some other member of the Rho family of Ras-related proteins²⁵. For Ras-GRF, this would mean that two independent exchange domains exist to activate distinct members of the Ras superfamily of proteins. The notion of a multifunctional releasing factor is supported by the analogous finding that Ras-GAP and a putative GAP (p190) for a member of this same Rho family of Ras-related proteins exist as a complex in cells²⁶. But when the N-terminal half of Ras-GRF containing the Dbl-like domain was expressed as a fusion protein with GST, it failed to enhance nucleotide exchange by *E. coli*-expressed CDC42Hs or by two other members of the Rho subfamily, Rac2 and RhoA (data not shown). It also had no effect on *E. coli*-produced RasH or RalA proteins. These negative results are not conclusive, however, because some nucleotide-releasing factors require their targets to contain

Antisera raised against a fusion protein between GST and a portion of the catalytic domain of Ras-GRF were used to detect Ras-GRF in cell extracts. When detergent extracts of rat brain were immunoprecipitated and then western blotted using the same antisera, a band of ~140K was observed (Fig. 4b, lane a).

1 CCAGCAGGGGCGAGTCGGGTGCCTCTCGGAGATGTTTAGTCGTCGAGGTCCTCTTGGCCCTCCAAGCACCATTGCAGAAAGCCATCCGACTGAACGATGCG
1 CACGTCGTGTCCCTGGGACTGCTGGCCCGAGAGACGGTACGCGCAAAGGCTACCTGAGCAAGAGGAGTTCGGACAACCCAAAATGGCAAAACAGGTGGTTTGGCGCTGCGACAACTG
100 H V V T S L G L L A G Q R D G T R K G Y L S K R S S D N P K W Q T K W F C L L L Q N L
11 CTCTTCTACTTCGAAAGTGACTCGAGCTCTCGGCCCTCGGGGCTCTACCTGCTGGAAGGAGCAGTATCTGCAAAACGCGATGCCCTCCCCAAGCGAGGACCTCTCCAAAGGAGTCCGACAAA
220 L F Y V F E S D S S S R P S G L Y L L E G S I C K R M P S P K R G T S S K E S D K
51 CAGCATCATTACTTCACGTGAACCTCTCCAATTGACAGCCAGAAAGTCCCTAGAGCTGAGGACCGATGACTCCAAGGACTGTGACGAGTGGGTGGCAGCGGATGTCTCGGCCAGCTCAAG
340 Q H H Y F T V N F S N D S Q K S L E L R T D D S K C D E W V A A I A R A S Y A K
91 ATACTGGCCACAGAGCTGAGGCGCTCATGCAAGAATCCTGCAAGCTGCTGAGGTCGGAGACAGAGAAGCCGCGGCTGAAGCGCTGCGACAGCAGCTCGAGGATGGCGAGGTCGAG
460 I L A T Q H Q A L M Q K Y L H L L Q V G V E T E K T V A K Q L R Q Q L E D G E V E
131 ATCCAGGCGCTGAAGCGAGAGATTGCAAACTGATCAAGGACATGAACGATTCAGTCAGTCCAACAGCTGGTTGACPECTGAGGATGAGGACGATGACATCAAGAAAATAAGAAAGGTACAG
580 I E R L K A E I A N L I K D N E R I Q S N Q L V A C P E D E D E S D I K K I K K V Q
171 AGTTCCTCTCGCGGATGCGTGTGCGCGCAAAGTGAAGAACATCATCCAGGACTACATCCGGTCTCTCATGCCGACAGCATGCGCAAGAGGAACAGGTGGTGTTCAGCATGCTGGAA
700 S F L R G W L C R R K W K N I I Q D Y I R S P H A D S M R K R N Q V F S M L E
211 GCTGAGGCGGAGTCAGTGCAGCAATACATCTCTTGCAAACTTTCTGGCCGCTCGCGCATGGCGCGGACTCTTAAGAAACCCCTATAACACATGACGACGTCAGCAGATCTTT
820 A E A E Y V Q Q L H L I L V N N F L R P L R M A A S S K K P P I T H D D V S S I F
251 CTGAACAGCTGAGACCATGATGTTCTGCGACAGATCTCTACCAAGGCTGAAGCGCGGTATGCGGACGCTGCCCACTGGTTGTGGCGGACCTGTTGCATCTCTGCTGCCAATGCTT
940 L N S E T I M F L H Q I F Y Q G L K A R I A S W P T T L V L L A D L F D I L L P M L
291 AACATCTACAGGAGTTCGTCGCAACCCAGCTACAGTCTCCAGATCTTACGACACTGCAAGCAAAACCGGAGCTTTGACAAGCTCCTCAAGCAGTATGAGGCGAAGCGCAGACTCGGAG
1060 N I Y Q E F V R N R N I Q I L A H C K Q N R D F D K L L Q Y E A K P D C E
331 GAGGCGACCTGGAGACCTCTCTCAGCTATCCAATGTTCCAGATCCCCAGGTACATCTGACACTCCATGAGCTGCTGGCCACACACCTCATGAGCATGTGGAGCGCAACAGCTGGAG
1180 E R T L E T F L T Y P M F Q I P R Y I L T L H E L L A H C T P H E H V E R N S L D
371 TATGCCAAATCCAACATGAGGAGCTGTCAGGCTCATGCAAGCAAGGACTGAGAGCTGAGCAAGAACTCCGCAAAAACCTGGCCATGAGCGTATGATCACCGAGGCTGTGAGATGCTCTC
1300 Y A K S K L E E L S R V M H D E V S E T E N I R K N L A I E R M I T E G C E I L
411 CTTGACACGCGGACGACCTTTGTGGCGCAAGGTTCCTCATCAGGTGCGCATGTGCAAAAAGGGAAGATCAACAAGGCGCGCTGGGTTCTGTCTCCCTTAAGAAAGAAAGGTGAGCGC
1420 L D T S Q T F V R G S L I Q V P M S E K G K I N K G R L G S L S L K K E G E R
451 CAGTGTTCCTGTTCTCCAAGCATCTCATCTGACCCAGAGGCTCTGGTAGCAAACTGCACCTAACAAGAAATGGCGTGATTCCCTCATTGACTGCACTCTACTGGATGATCCAGAA
1540 Q C F L F S K H L I I G A T R G S G S K L H L T K N G V I S L I D C T L D D P E
491 AACATGGATGATGACGGGAAGGACAAGAGGTAGTACCTGAGCTTTAAGATTTGGGTGGAGCGCAAAAGGATTCGCCACVITCAGAGTCATCCGTGGCGCTCATCCGAGCGAGAGAAG
1660 N M D D D G K G Q E V D H L D F K I W E V C K D S P P F T V I L V L A S S R Q E K
531 CGCGCATGAGCAGTGCATCATCCAGTCGCTGGATATCTCCGCTGCAACGGGCTCATGATGAATGCCTTTGAAGAAAATTCGAAGTCACCGTGCAGCAGATGATCAAGTCTGATGCT
1780 A A W T S D I I Q C V D N I R C N G L M M N A F E E N S K V T V P Q M I K S D A
571 TCCTTATATGTGATGTGACATTCGCTTCAGCAAAACCATGAATCTTGCAAAAGTCTGAGATCCGCTATGCCAGCTGAGGCGCTGCTGGAAGGCGCTGACTGATCTTCGCTTC
1900 S L Y C D D V D I R F S K T M N S C K V L Q I R Y A S G V T G R L L E R L T D L R F
611 CTGAGTATTGACTTTCTCAACACTTCCTGACCTCTCTGAGCTCTTACCGATGCTGTTGGTGGCTAGACAAGCTGATCAGCATCTACAAAAAGCCCTACTCGCATTCCTGCGCAGG
2020 L S I D F L N T F L H S Y R V F T D A V V V L D K L I S I Y K K P I T A I P A R
651 TCATCGAACTCTGTTCTCCAGTAGCCACAACCAAACTCTGTACGGAGATGCCCCAAGTCGCTGTGCCAGCGCGCAAGTTCTCTCGCGCGCGCCCTTGGCCATCGGCATCTG
2140 S L E L L F S S S H N T K L L Y G D A P K S P R A S R K F S S P P L L A I G T S
691 TCCCGACTCGCGCGCGGAAGTTGTCTCTCAACATTCCTCATATCAGGCGGCAAGGCGCTGGAATGGCTTGCCTGGGTGCCCCCTCCGACGGCTACACCAACATCACTACCTCGCCATA
2260 S P V R R R K L S L N I P I T I T G K A L E L A S L G C P S D G Y T N I H S P I
731 TCTCCCTTCGGCAAAACCGCTGGACACCGCAAGCTGTGTGTGGCCAGCAGCTTGACACAGAACCGCGGAGGAGATTGATGACCATTCTAGAGGAGTCTACAGGCTTCAGGAAGCGG
2380 S P F G K K T T L D T S K L C V A S S L L R T P E E I D M T T L E E S S G F R K P
771 ACCTCAGACACTCTGAAGAAGATCTGATGATGACGAGCTGATGAGACACAGAAAGTGTCTCCACCAACACCGAAATCATTGAGAAACAGAACTACTCAAGAGTTCCCACTCTTT
2500 T S D I L K E E S D D D Q S D D V D D T E V S P P T P K S F R N R I T Q E F P L F
811 AACTACAACTGGAATCATGATGACATGTGCGGATCTGATGGACAGTAAACCGAGCCCTCTGCTAGCTACTCTGCTTTGGCATAGGCACTGAGGAGCGCAATGAAGGCGCGCAAAAC
2620 N Y N S G I M M T T C R D L M D S N R S P L S A T S A F A I A T A G A N E S A N
851 AAGGAGATATCGAAGGATGCTCTTGCCCAACACAGGTTATCCCTGACCCAGAGAAATATCGACAAGAGTTCGTGATCCGAGCGCGCCCAACACCGTGTACTGATGTGTCGGC
2740 K E I Y R R M S L A N T G Y S D Q R N I D K E F V I R R A A T N R V L N V L R
891 GACTGGGTCAACAGCACTCCGAGGACTTTGAACTGACGACCTCTCAATACCAAGGTGATCTGCTTTTGGAAAGAGGTCATGATGACCCAGCACTCTTACCAAGACGCGAAAGGCA
2860 H W V T K H S Q D F E T D D D L L K Y K V I C F L E E V M H D P D L L P Q E R K A
931 CGAGCCAACTCATGAGGACTGTGACCCAGGAAGAAATACTGAAACCACTAGATGCTGGATGAGCTTCTACTAATGACGAGGAGGTGAGAGCTGAGGCTTGGAAACCACTAGCC
2980 A A N I M R T L T Q E E I E N H S M L D E L L M T E G V K T E P F E N H S A
971 ATGGAGATAGCAGAGCTGACCTGCTGGATCACTTGTCTTCAAGAGTATTCTTATGAGGAATTTTGGCCAGGCGTGGATGAAGGCGAGATAAGAATGAAGGACACCTTACATT
3100 M E I A E Q L T L D H L V F K S I P Y E F F G G W M K A D K N E R T P Y I
1011 ATGAAAACCCAGACATTTCAACCATATCAGTAACCTGATGCTTCAGAAATTTCCGAAACAGGAGGTCAGTGAAGGCGCAAGCACCATTGAGAAAGTGGGTGGCTTGTCCGACATT
3220 M K T T R H F N G I T H L I A S E I L R N E E V S A R A S T I E K W V A V A D I
1051 TGCCGTGCTGCACTCAACTACAACTGTGCTGGAGATCACTTCTCCATCAACCGAGCGCAATCAACCGACTCAAGAAGCACTGGCTCAAAGTTTCTAAGCAGCAAAATCTCTGTTT
3340 C R C L L H N Y N A V L E I T S S I N R S A I F R L K K T W L K V S K Q T K S L F
1091 GACAAGCTCCAAAGCTTGTGTCATCAGATGCGCGAATTAAGAACTCAGAGAACTTTGCAAAATTTGATCCACCTGTGCTCCTTACCTGGGGATGTACCTGACCGCACTTGGCATT
3460 D K L L G A A G G A A C C C A A T T C A C A G G A C G G C T G G T C A A C T T C C A A G A T G A G G A T G A T C C C A T A T T A T C C G G A G A T C C G A C T A C T T A C A A A T C G A G
1131 C T C G A G G A A G G A A C C C A A T T C A C A G G A C G G C T G G T C A A C T T C C A A G A T G A G G A T G A T C C C A T A T T A T C C G G A G A T C C G A G T A C T T A C A A A T C G A G
3580 L E E G T P N Y T E D G L V N F S K M R M I S H I R E I R Q F Q Q T T Y K I E
1171 C C C C A G C C A A A G G T A A C T A G T A C T T A G T G A T G A A A C C T T G T G T G G A C G A A A G T C T G A T G A G G C T C C C T C G A A T T G A A C C A A A C T C C C C A C T A G A
3700 P Q P K V T Q Y L V D E T F V I D D E S I L Y E A S L R I E P K L P T
1211

METHODS. Two degenerate oligonucleotides (5'-CCIATT/CC/TT/CA/CGG/ACUIIA/GG/AAAA/GACIUGG-3' and 5'-TAIACIICCIAGG/AAAGGIACG/ACA-3', where I refers to inosine) were designed to code for all possible peptides in two conserved regions of *S. cerevisiae*, CDC25 and SDC25 proteins as well as *S. pombe* Ste6. The amino acids used were 1,407-1,415 and 1,454-1,461 in *S. cerevisiae* CDC25 (ref. 16), 1,054-1,062 and 1,101-1,108

in *S. cerevisiae*, SDC25 (ref. 17) and 764–772 and 810–817 in *S. pombe* Ster 6 (ref. 18). The oligonucleotides were used in a PCR reaction with DNA from a λ GT11 rat brain cDNA library (gift from D. Chikaraishi) as template. The 159-bp fragment generated was subcloned into pBluescript and used as a probe to screen a λ GT10 rat brain cDNA library (Clontech). Multiple overlapping clones were isolated and both strands were sequenced. About 400 bp of 3' untranslated sequence including poly(A)⁺ and ~600 bp of 5' untranslated sequence are not shown.



FIG. 2 Sequence similarity between Ras-GRF and other proteins. Sequences were compared to those in the Genbank database with the FASTA program GCG software package. The protein sequences were aligned using the

BESTFIT program. Dashes between amino acids represents identical sequences, whereas double dots signify conservative changes grouped as follows: D/E; S/T; I/L,V/M; A/G; F,W,Y; N/Q; H/R,K; C; P.

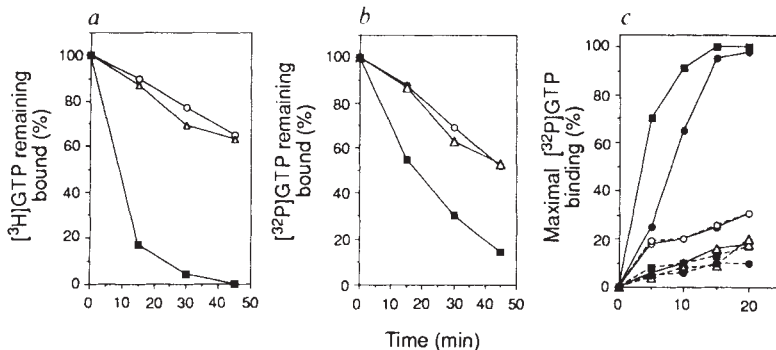
This size is consistent with that predicted from the cDNA sequence. The band was specific for Ras-GRF, because a comparable band was not observed when pre-immune sera was substituted in the immunoprecipitation (Fig. 4b, lane b) or the western blot (data not shown). Furthermore, preabsorption of immune sera with the antigen blocked the detection of Ras-GRF (lane c), but preabsorption of sera with GST alone did not (lane d). Finally, consistent with northern blot data, p140^{Ras-GRF}

was not detected in extracts of other tissues such as liver (lane e).

Fractionation of brain homogenates demonstrated that Ras-GRF is localized in both the particulate and cytosolic fractions (Fig. 4b, lanes f and g). The activation of Ras by Ras-GRF probably occurs by the particulate-associated releasing factor, because Ras p21 must be localized to the plasma membrane to function²⁷. More importantly, a dominant inhibitory form of

FIG. 3 Effects of recombinant GST-p55^{Ras-GRF} on guanine nucleotide binding by RasH p21. **a**, GDP dissociation. Samples of GST-p55^{Ras-GRF} (■), GST (○) or buffer (△) were added along with excess unlabelled GTP to incubations containing [³H]GDP-bound RasH p21. The rate of dissociation of [³H]GDP was measured. **b**, GTP dissociation. Same as in **a**, except that the dissociation of [³²P]GTP was measured. **c**, GTP binding. Samples of GST-p55^{Ras-GRF} (solid lines) or GST (dashed lines) were added to incubations containing RasH p21 (■), RasN p21 (●), RalA (△) or CDC24Hs (○) and the rate of [³²P] binding was measured.

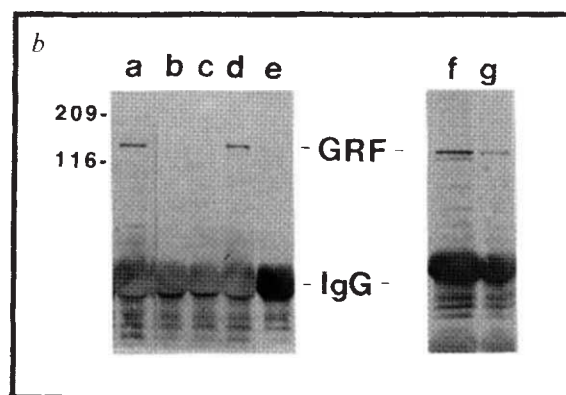
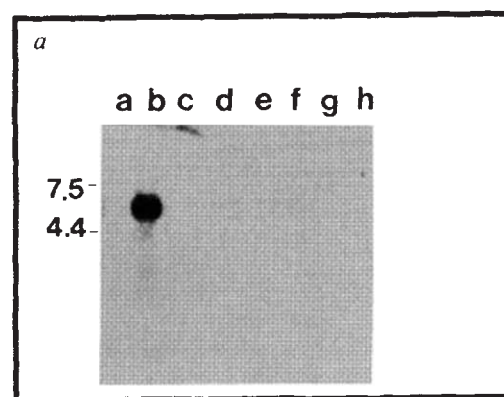
METHODS. RasH p21 and RalA p24 were purified from *E. coli* as previously described³¹. DNA coding for the C-terminal 456 amino acids of Ras-GRF was cloned into the *Eco*RI site of pGEX-3 (Pharmacia). After transformation into JM101 and induction with isopropyl β -D-thiogalactoside, GST-p55^{Ras-GRF} was purified from the soluble fraction of cell extracts with glutathione agarose beads (Sigma) as described by the manufacturer. **a** and **b**, RasH p21 (10 pmol) was incubated with either [³H]GDP (100 pmol, 10.5 Ci mmol⁻¹) or [³²P]GTP (100 pmol, 65 Ci mmol⁻¹) for 60 min at 32 °C in 160 μ l binding buffer A (20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), 100 mM NaCl, 40 μ g ml⁻¹ bovine serum albumin). Then, nonradioactive GTP (final concentration, 100 μ M) plus beads (40 μ l) containing either bound GST (2 μ g) or GST-p55^{Ras-GRF} (2 μ g) were added. At the indicated times, 40 μ l of the reaction were removed, the beads were pelleted by centrifugation at 10,000g for 1 min, and 15 μ l of the solution was filtered through nitrocellulose. Radioactivity associated with the filter was quantified by scintillation counting. **c**, The reaction mixture (170 μ l) contained binding buffer A



and 100 pmol RasH p21, RasN p21, RalA p24 or CDC42Hs. The reaction was started at 32 °C by the addition of [³²P]GTP (final concentration 1.5 $\times$ 10⁻⁷ M, 65 Ci mmol⁻¹) and 30 μ l of beads containing GST or GST-p55^{Ras-GRF} (2 μ g). At the indicated time, 45 μ l of the reaction were removed and centrifuged at 10,000g for 1 min to remove the beads. The amount of [³²P]GTP bound to each protein was determined as described above. EDTA (5 mM) was added to some samples as a positive control to confirm that RalA and CDC42Hs could indeed bind GTP in these experiments. In **a**, **b** and **c**, each data point is the average of duplicate determinations, and is expressed as the percentage of maximal counts bound in the presence of protein and EDTA. The results shown are representative of at least three independent experiments.

FIG. 4 Northern and western blot analysis of p140^{Ras-GRF} expression. *a*, Northern blot. Poly(A)⁺-selected RNAs from a variety of mouse tissues were hybridized with a [³²P]-labelled p140^{Ras-GRF} probe coding for a portion of the protein's catalytic domain and autoradiographed. The tissues included heart (lane a), brain (lane b), spleen (lane c), lung (lane d), liver (lane e), skeletal muscle (lane f), kidney (lane g) and testis (lane h). *b*, Western blot. Detergent extracts of rat brain (lanes a-d) or rat liver (lane e) were immunoprecipitated with anti-GST-GRF antiserum (lanes a, c, d and e) or preimmune serum (lane b) and then western blotted with the same anti-serum (lane a, b and e) or the anti-serum pre-absorbed with either GST-p55^{Ras-GRF} (lane c) or GST only (lane d). The particulate (lane f) and soluble fractions (lane g) of rat brain extracts homogenized with non-detergent containing buffer were immunoprecipitated with anti-GSF-GRF antisera, and western blotted with the same anti-sera.

METHODS. *b*, A fusion protein of GST and a fragment of Ras-GRF containing amino acids 775-1,032 was made by cloning a unique Ras-GRF cDNA clone containing these sequences into the *EcoRI* site of pGEX-2. The fusion protein was purified from the soluble fraction of bacterial lysates with glutathione beads and used to immunize rabbits. The resulting anti-serum and pre-immune serum control were used at a dilution of 1:100 to immunoprecipitate Ras-GRF from rat brain extracts. Extracts were prepared by homogenizing rat brain in RIPA buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4 and 1% aprotinin). Protein (1 mg) from the resulting supernatant was used for each immunoprecipitation. The immunoprecipitate was collected on Protein A beads and then run in an SDS polyacrylamide gel. The proteins in the gel were transferred to nitrocellulose and probed with the same anti-Ras-GRF antiserum diluted 1:500. The immune-complex was detected with goat anti-rabbit IgG coupled to alkaline phosphatase. For preabsorption experiments, glutathione beads containing ~5-10 µg of either GST-p55^{Ras-GRF}, or GST were incubated with antiserum for 60 min at 4 °C, before their use in western blots. Tissue fractionation. Rat brains were homogenized in lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1% aprotinin) with 10 strokes with a Dounce homogenizer. Unbroken cells were removed by three successive centrifugations at 10,000g for 15 min at 4 °C. The resulting supernatant was separated into soluble and particulate fractions by centrifugation at 100,000g, 4 °C for 60 min. The particulate fraction was solubilized in RIPA buffer and the soluble fraction was diluted with an equal volume of 2 × RIPA buffer before the immunoprecipitation. *a*, A multiple tissue poly(A)⁺ blot was purchased from Clontech. It was prehybridized at 42 °C for 3 h with 5 × SSPE (0.75 M NaCl, 50 mM NaPO₄, pH 7.4, and 5 mM Na₂ EDTA), 10 × Denhardt's solution, 100 µg ml⁻¹ denatured and sheared salmon sperm DNA, 50% formamide, and 2% SDS. The blot was then hybridized at 42 °C



for 12 h in the same buffer containing a [³²P]-labelled probe of Ras-GRF (nucleotides 2,370-3,870) (10⁸ c.p.m. µg⁻¹; 10⁶ d.p.m. ml⁻¹). After washing in 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) and 0.05% SDS the blot was washed again in 0.1% SSC at 50 °C for 40 min. The blot was autoradiographed for 2 days. Qualitatively similar results were obtained after 7 days of autoradiography. Reprobing the same blot with an actin probe showed similar levels of RNA were present in all lanes of the blot.

Ras, which seems to inhibit endogenous Ras function by competing for Ras-GRF²⁸, must itself be in the plasma membrane to display inhibitory activity²⁹. Thus, activation of Ras in cells may depend on mechanisms that regulate the distribution of Ras-GRF between the cytosolic and membrane compartments.

The p140^{Ras-GRF} may be the same as an exchange factor activity previously detected in brain cytosol⁹. Although activity of the latter could not be detected in crude particulate fractions, its estimated size was similar to that shown here for Ras-GRF. In contrast, a different type of guanine nucleotide-releasing factor, which acts on RasK p21, has been cloned and termed GDP-dissociation stimulator. Unlike Ras-GRF, this GDP-dissociation stimulator requires Ras to be post-translationally

processed, does not effect RasH p21, but does affect other Ras-related proteins³⁰.

Ras proteins participate in signal transduction pathways that regulate cell proliferation in some cells and the expression of differentiated function in others²⁷. Cell-specific Ras function may be mediated in part by distinct Ras-GRFs. For example, the unique properties of p140^{Ras-GRF} may allow Ras in brain cells to be activated by unique upstream signals. Moreover, the presence of a putative GRF domain for another member of the Ras superfamily in p140^{Ras-GRF}, suggests that multifunctional GRFs may contribute to the formation of tissue-specific signalling complexes that coordinate downstream signals emanating from multiple signalling molecules. □

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